ACTORS IN A DYNAMIC SCENE

On the interplay between macrophages and local mediators in the fibrotic liver

Marlies Schippers

The work described in this thesis was performed at the Department of Pharmacokinetics, Toxicology and Targeting, University of Groningen, The Netherlands. The research project was financially supported by a VICI grand from the Netherlands Organisation for Scientific Research (NWO) and the Dutch Technology Foundation STW (grant GFA 6797).

Cover: Illustration of the change in the hepatic cellular composition.

ACTORS IN A DYNAMIC SCENE
on the interplay between macrophages and local mediators in the fibrotic liver

ISBN: 978-90-367-6119-2
ISBN electronic version: 978-90-367-6118-5

Cover design: Marc Daalmans
Layout: Marlies Schippers and Djoek Vogel
Printing: Off Page, www.offpage.nl

RIJKSUNIVERSITEIT GRONINGEN

ACTORS IN A DYNAMIC SCENE

On the interplay between macrophages and local mediators in the fibrotic liver

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
maandag 29 april 2013
om 14.30 uur

door

Anna Margaretha Theodora Schippers

geboren op 6 november 1978

te Rheden

Promotor:	Prof. dr. K. Poelstra
Co-promotor:	Dr. L. Beljaars
Beoordelingscommissie:	Prof. dr. H.J. Haisma
	Prof. dr. H. van Goor
	Prof. dr. M. Schmidt

Paranimfen: Eduard Post
Djoek Vogel

The printing of this thesis was financially supported by grants from:
AM-Pharma University of Groningen, Groningen Institute for Drug Exploration (GUIDE).

Voor mijn lieve stoertjes

Contents

CHAPTER 1

Introduction and scope of the present thesis

INTRODUCTION

During injury of the liver by for instance toxins, autoimmune diseases, drugs or alcohol, damaged hepatocytes secrete various inflammatory cytokines that activate the resident macrophages (kupffer cells, KC) and hepatic stellate cells (HSC). While a short, inflammatory response is beneficial to restore the liver architecture, uncontrolled continuation of this process may lead to liver fibrosis. Liver fibrosis is characterized by excessive tissue remodelling which results in an abundant deposition of extracellular matrix (ECM). The signals to terminate this process fail and progressive scarring of the liver leads to hepatocellular dysfunction and finally death. Up to now fibrosis is untreatable, partly due to its multi-cellular character and complex cell-cell interactions.

In the liver, the interplay between macrophages and HSC greatly determines disease activity. HSC become activated in response to mediators such as transforming growth factor β (TGFβ) and platelet derived growth factor BB (PDGF-BB) and transform into ECM-producing myofibroblasts[1,2]. In the past decades, HSC received most attention as the key player in liver fibrogenesis, but it is becoming increasingly clear that KC, as well as the newly recruited macrophages in the liver also do play an important role in the pathogenesis of liver fibrosis via activation and regulation of HSC activities[3,4].

AIM OF THE THESIS

The studies presented in this thesis aimed to investigate the role of endogenous mediators Prostaglandin E_2, Interferon gamma and Alkaline phosphatase in the acute and chronic phase of liver fibrosis. Thereby we focused on the localization and accumulation of different macrophage subtypes during fibrogenesis. In addition, we aimed to examine the intracellular pathway of PGE_2 in the fibrotic liver and used our drug targeting approach to investigate which cell type and which pathway is responsible the antifibrotic effect of PGE_2.

MACROPHAGE SUBSETS AND FUNCTION

Macrophages are derived from blood monocytes and are regulated by macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) from the bone marrow[5,6]. Macrophages are part of the first line host defence but are also involved in the regulation of wound healing and tissue remodelling. To perform these diverse functions, macrophages consist of at least two subsets: the classical activated macrophages (M1 phenotype) and the alternative activated macrophages (M2 phenotype)[5-8]. Both phenotypes are activated by different mediators and are endowed with different activities.

The M1 macrophages have a microbicidal or tumoricidal capacity and are activated by TNFα, IFNγ and LPS. They secrete pro-inflammatory cytokines such as IL1ß, IL6, TNFα, IL12 and IL23, promoting strong Th1 and Th17 immune responses[7,9,10]. M1 macrophages express opsonic receptors including Fc-receptors and Toll-like receptors (TLR), resulting in enhanced binding capacity for the process of phagocytosis[8]. In addition, this macrophage subtype produces reactive oxygen and nitric oxide (NO) leading to damage of the surrounding tissue and also produces metalloproteinases (MMPs) that degrade scar tissue[11]. The pro-inflammatory activity of the M1 macrophages is counterbalanced by M2 macrophages. In

contrast to M1 macrophages, this phenotype has immunosuppressive actions via the release of anti-inflammatory cytokines like IL4, IL13, promoting Th2 immune responses[7,9,12]. M2 macrophages have a high mannose- and scavenger-receptor expression[13-15] and produce high levels of arginase-1, to counteract NO production[11]. This phenotype promotes wound healing activities by fibroblast activation and secretes tissue remodelling enzymes including tissue inhibitors of metalloproteinase (TIMPs) and precursors for ECM proteins[16-18]. One subpopulation of M2 macrophages are the M2-like macrophages, also called M2c or regulatory macrophages. Regulatory macrophages are generated in response to prostaglandins, glucocorticoids, immune-complexes and IL10[6,8,19], have anti-inflammatory activities by the secretion of IL10, TGFβ and PGE_2[5] and they promote Treg responses. In contrast to the M1 and M2 macrophages, regulatory macrophages have a low expression of MHCII[5]. It is still unclear whether Treg cells have pro-or anti-fibrotic actions, since both activities are described in literature[20-25]. In summary, all these macrophage subtypes are needed to participate in a homeostatic process; to survive from attacks, regulate immune-responses and restore damaged tissue. However, deregulation of each of these subtypes leads to patho-physiological responses including persistent inflammation, tissue damage and fibrosis (Figure 1).

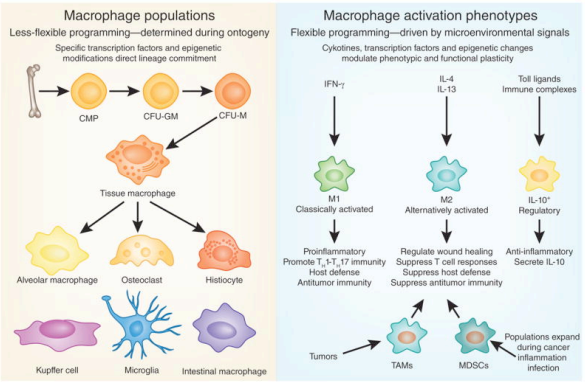

Figure 1; Macrophage populations and functional subsets. Macrophages can be subcategorized into specific populations on the basis of their anatomical location (left) and functional phenotype (right). Tissue-resident macrophages include alveolar macrophages (lungs), histiocytes (interstitial connective tissue), osteoclasts (bone), microglia (brain), intestinal macrophages, Kupffer cells (liver) and so on. Mononuclear phagocyte subpopulations in the circulation can also differentiate into tissue macrophages after entering different anatomical sites; when activated by the appropriate stimuli, these cells differentiate into various subsets with distinct phenotypic and functional characteristics. *This figure is republished with permission*[26].

MACROPHAGES IN INFLAMMATION AND FIBROSIS

During acute injury, the rapid responsive coagulation cascade and the wound repair process are tightly intermingled[27]. Thrombin is known to promote activation and proliferation of HSC and thereby fibrogenesis via protease-activated receptors (PAR). These PARs are mainly expressed in the liver and several studies show that PAR levels increase along with the progression of liver fibrosis[27-29]. In addition to activation of the coagulation cascade, the recruitment of inflammatory monocytes and neutrophils is also important for the wound-healing process[12,29]. During acute injury, Th1-related cytokines activate M1 macrophages, which secrete, together with hepatocytes and HSC, matrix metalloproteinases (MMPs) to allow efficient tissue access for inflammatory cells at sites of injury and promote tissue remodelling[7,9,30,31]. After this migration, anti-inflammatory, Th2-related cytokines activate and recruit M2 macrophages and monocytes to remove cell debris. So, the M2 macrophages counterbalance the M1 response and stimulate HSC activation and wound healing[7-9,32].

The balance between inflammation and wound healing by respectively M1 and M2 macrophages is essential to mediate resistance to pathogens and restore liver tissue. Macrophages activated by LPS and Th1 cytokines ("classical macrophages") fulfil critical actions in the initiation of inflammatory responses and tissue remodelling. However, in established fibrosis these macrophages have anti-fibrotic functions because of their MMP production and ECM degrading activity. Classically activated macrophages are thus characterized by pro-inflammatory, but anti-fibrotic activities. In contrast to the M1 macrophages, M2 macrophages are commonly found in later stages of disease and actively regulate ECM rearrangement processes[30]. Macrophages can directly affect fibrogenesis by the production of HSC-activating growth factors such as TGFβ and PDGF[11] and, moreover, they produce Tissue Inhibitors of Metalloproteinases (TIMPs), a pro-fibrotic mediator that inhibits ECM turnover. In the acute phase of injury, M2 macrophages prevent overshooting of Th1 responses and inhibit further exacerbation of tissue damaging inflammatory responses that lead to scarring. Alternatively activated macrophages thus have anti-inflammatory, but pro-fibrotic activities.

The group of Iredale showed opposite roles of macrophages in the acute and repair phase of fibrogenesis which they related to MMP13-producing scar-associated macrophages[3,4]. These opposite roles of macrophages in inflammation and fibrosis may be ascribed to the fact that the population of M1 and M2 macrophages is different in both phases of injury. We therefore studied the localization and expression of M1 and M2 macrophages during acute and chronic fibrogenesis, and studied changes in macrophage polarization during liver regeneration, as described in **chapter 2**. We observed that during fibrogenesis, macrophages migrate from the parenchymal to the fibrotic bands and found a rearrangement of the macrophage population within the liver.

BIOLOGICALS AND ACTIVATION OF HSC AND MACROPHAGES

The biological activity of macrophages is tightly controlled by cytokines, oxidants, lipid mediators and growth factors released by the different types of macrophages, but whether the balance between the

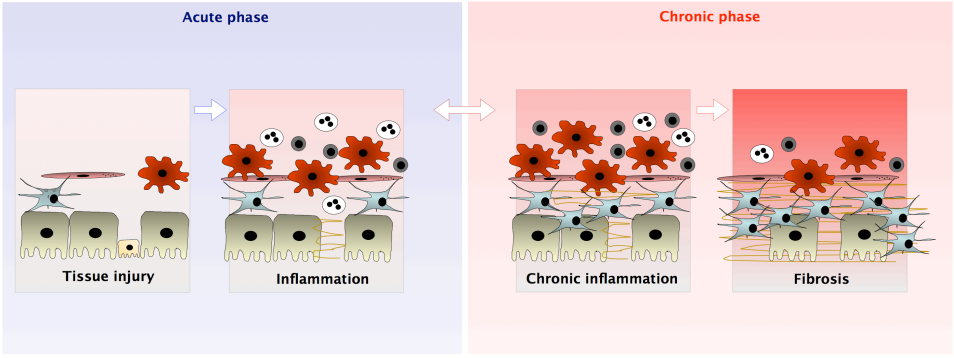

Figure 2; Schematic overview of the acute and chronic phase after liver damage. Tissue injury leads to activation of platelets and recruitment of neutrophils. Kupffer cells and HSC become activated leading to wound-healing processes and restoration of the damaged area. When injury persist, chronic inflammation leads to an uncontrolled wound-healing process, characterized by an abundant deposition of extracellular matrix produced by HSC.

different subtypes of macrophages is affected by these mediators is unknown. The chronic nature of liver fibrosis, which is a disease process that may take decades to reach end-stage liver failure, suggests an important role for endogenous anti-fibrotic mediators in this process. The most well known endogenous anti-fibrotic mediators include PGE_2 and IFNγ. These mediators are produced by many cell types within the fibrotic liver such as the macrophages, HSC and hepatocytes and they may regulate the fibrotic process at different levels.

Prostaglandin E_2

Prostaglandin E_2 is a potent lipid mediator derived from the oxidation of arachidonic acid by cyclooxygenases (COX-1 and COX-2) and is a well-known regulator of different (patho) physiological processes such as blood flow, fever, inflammation, fibrosis and cancer, in various organs. The diverse effects of PGE_2 can be ascribed to the fact that PGE_2 acts via its G-coupled protein receptors EP1 to EP4 which are present on multiple cell types and affect second messenger cyclic AMP (cAMP) in different ways[33,34].

Although PGE_2 is well-known for its role in inflammation and cyclooxygenase products are known to play a role in many diseases including liver fibrosis, its mechanism of action during fibrogenesis has never been extensively studied *in vivo*. This lack of clarity *in vivo* is most likely due to its pleiotropic effects and its poor pharmacokinetic profile; it is rapidly cleared by the liver and kidneys, inactivated by plasma proteins or metabolized and oxidized in plasma[35]. Very few publications report on the intrahepatic effects of PGE_2 *in vivo*, i.e. on the accumulation of triglycerides in hepatocytes induced by PGE_2 during ethanol-induced steatosis[36], the inhibitory effect of PGE_2 on the acute phase response[37] and the reduced collagen deposition after treatment with a stable PGE_2 analogue[38]. In cultured fibroblasts, PGE_2 exerts anti-proliferative and anti-fibrogenic effects through cAMP activation by binding to its EP2 and EP4 receptor[39-42].

PGE_2 is also known to regulate immune responses[43], suppresses cytokine production[44], induces iNOS expression[45], regulates anti-inflammatory actions in alveolar macrophages[46] and inhibits macrophage maturation in the bone marrow[47]. So, PGE_2 profoundly influences inflammatory processes by exerting both pro-inflammatory and anti-inflammatory actions on macrophages[48-51]. However, its effect on the polarisation of macrophages into M1 and M2 phenotypes during fibrogenesis *in vivo* is unclear. *In vitro*, PGE_2 was found to induce an M2 phenotype in tumor-associated macrophages[52], and in human mesenchymal stem/ stromal cells endogenous PGE_2 stimulated polarisation of macrophages into the M2-subtype[53].

Interferon gamma

Interferon gamma (IFNγ) is a pro-inflammatory cytokine which is produced by Th-1 activated immune cells[9]. IFNγ drives the macrophage polarization into the M1 subtype and promotes strong pro-inflammatory activities[6,32]. During fibrogenesis, IFNγ inhibits ECM deposition, HSC-activity and proliferation *in vitro* and *in vivo*, and is therefore a potent mediator for anti-fibrotic activities[54]. So, besides the potent antifibrogenic effects of IFNγ, this cytokine is endowed with strong pro-inflammatory activities, with the macrophage as a major target cell.

Chapter 3 subsequently describes the effect of PGE_2 and IFNγ on the M1/M2 balance during the acute and chronic phase of fibrogenesis. We found that both mediators, directly or indirectly, affected the

macrophage balance significantly, which was associated with significant changes in fibrogenic activity. Most surprisingly, both mediators affected macrophages and fibrogenesis in different ways in different phases of the disease. In addition, a significant correlation was found between changes in the number of M2 wound-healing macrophages and collagen deposition. This study illustrates the importance of the M1/M2 balance in different stages of disease and also identifies PGE_2 and IFNγ as important mediators affecting disease activity, both steering responses towards homeostasis.

EPAC-1 AND PGE2

The next step was to unravel the intracellular pathway of PGE_2, that would explain the anti-fibrotic effects of this mediator in fibrotic livers. PGE_2 is known to regulate its effect via modulation of cAMP activity[39]. In various cell types, PGE_2 activates cAMP signaling via its EP2 and EP4 receptor, but it can also act as a negative effector by inhibition of cAMP signaling via its EP3 receptor, implicating an important regulatory effect in many (patho) physiological processes.

Besides the classical cAMP effector Protein Kinase A (PKA), Exchange Protein Activated by cAMP (Epac) was recently discovered as a new downstream mediator for cAMP-mediated effects[55]. Epac is a guanide nucleotide exchange factor (GEF), specific for the Ras family[56]. It is involved in the regulation of several cellular key processes such as calcium handling, neural signalling, inflammation, cell proliferation and migration, by promoting the exchange of GTP and GDP in the GTPase cycle[56,57](Figure 3). Two isoforms of Epac are now identified; Epac-1, which is reported in the heart, vasculature, brain, kidney and lungs, and Epac-2, mostly found in brains and adrenal glands[57].

A reduced Epac-1 expression by TGFβ was recently found in cultures of hepatic stellate cells, suggesting a role for this mediator during fibrogenesis. In addition, treatment of fibroblasts in cultures was found to influence both cAMP effectors PKA and Epac, however, little is known on Epac-1 levels during liver fibrosis *in vivo*. We therefore investigated the effect of PGE_2 on fibrogenesis and the mechanism of action of this cyclooxygenase product in a chronic CCl_4-induced mice model for liver fibrosis, as outlined in **chapter 4**. *In vivo*, anti-fibrotic effects of PGE_2 were observed which was associated with significant changes in Epac-1 expression levels within the liver. Moreover, we investigated the effect of COX-2 inhibition on collagen deposition and changes in the Epac-1 signalling pathway and found the opposite effects as compared to PGE_2. Detailed insight in this process is essential to understand the long term effects of COX inhibitors during fibrogenesis, which is relevant to millions of people world-wide. Of note,, also in the human liver, fibrosis was associated with changes in Epac-1 levels, identifying this signalling pathways as a potential new target for anti-fibrotic therapies.

DELIVERY OF PGE_2

As described before, PGE_2 is an important regulator in physiological processes in a variety of cells because of its action on EP receptors, expressed on almost every cell. In the liver, PGE_2 is known to regulate glycogenolysis in hepatocytes[58,59], the release of inflammatory mediators in Kupffer cells (KC)[60,61] and activation of hepatic stellate cells (HSC)[42,62,63]. In fibroblasts of different origin, activation of cAMP via EP2 and EP4 receptors revealed a role for Epac-1 in the proliferation and activation of these cells[40,42,64,65]. In addition, PGE_2 regulates antigen presenting cell functions via EP2 and EP4, and suppresses cytokine pro-

duction by macrophages via the EP4 receptor[44]. In muscularis resident macrophages, PGE_2 induces iNOS expression via EP2 and EP4[45] and also in alveolar macrophages anti-inflammatory actions were observed via activation of EP2 and EP4[46]. In hepatocytes, the EP receptors are involved in the proliferation of these cells[66,67]. Treatment of fibrotic rats with a stable analogue of PGE_2 induced lower collagen expression levels compared to untreated animals[38]. However, it is still unclear which cell type is responsible for this effect en the mechanism behind it.

Since fibrogenesis is a multi-cellular process and Epac-1 proteins are also expressed in several non-resident cell types including monocytes, macrophages, B and T cells and other blood cells[56] we aimed to investigate which cell type in the liver is the effector cell of the PGE_2-mediated effects *in vivo*. The cell-specific drug delivery approach described in **chapter 5** served to gain a better understanding of the signaling pathways that mediate PGE_2-induced anti-fibrotic effects *in vivo*. PGE_2 was coupled to cell-specific carriers directed at either hepatic stellate cells, Kupffer cells or hepatocytes and we subsequently evaluated their effect on fibrogenesis and the cAMP mediators Epac-1 and PKA in a chronic CCl_4-induced model for liver fibrosis in mice.

TARGETING CONCEPT

We used drug carriers consisting of a core protein, in our case Human Serum Albumin (HSA), to which targeting devices were coupled. Lactosylated albumin (LacHSA) was used as a carrier to reach the hepatocytes. The galactose-moiety of lactose is a ligand for the asialoglycoprotein receptor, which is selectively expressed on hepatocytes[13,68]. To reach the macrophages we used a carrier consisting of mannose groups coupled to human serum albumin (manHSA). The mannose-receptor is abundantly expressed on KC[13-15]. In addition, we used the PDGFβ-receptor as a target to reach activated HSC since this receptor is abundantly expressed on these particular cells[69,70]. We coupled a PDGFβ-receptor recognizing cyclic peptide to human serum albumin (pPBHSA) and used this carrier to deliver PGE_2 to the activated

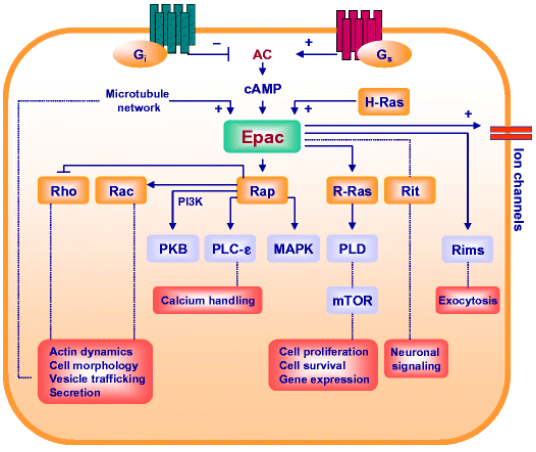

Figure 3; Epac: effectors and biological functions. Schematic representation of the Epac signaling pathway. Second messenger-generating receptors involved in activation of Epac are indicated; small GTPases are shown in orange, other effectors are shown in blue, and distinct cellular responses are indicated in salmon. AC, adenylyl cyclase; PLD, phospholipase D; PLC, phospholipase C; mTOR, mammalian target of rapamycin; PKB, protein kinase B. Whereas Epac2 is recruited by activated H-Ras to the plasma membrane, the GEF activity of Epac1 is regulated by LC2 MAP1A, a microtubule-associated protein. Signaling of various GPCRs, receptor tyrosine kinases and integrin receptors may converge on the level of the diverse effectors of Epac proteins (not shown) *This figure is republished with permission*[56].

HSC[71,72]. All three carriers are summarized in table 1 together with their cell-specificities that have been demonstrated *in vivo* in previous studies[13,15,54,71,72].

PGE_2 was subsequently covalently coupled to the backbone of HSA of the different carriers via an amino binding. The PGE_2-conjugates bind to their respective target receptors, are internalized, and subsequently degraded in the lysosomes[68]. After degradation of the protein-based carriers, PGE_2 is released from the carriers. Its lipophilic nature ensures release from the lysosomal compartment and PGE_2 can induce its pharmacological effect in the target cell. Local delivery with the designated target cell mimics the local production of PGE_2 within the diseased liver. Our cell-specific delivery approach revealed that the HSC is the main target cell for PGE_2-mediated antifibrotic effects and the Epac-1 signaling pathway within this cell-type appears to be responsible for this effect.

Tabel 1; Overview of the characteristics of the carriers

Targeting device (coupled to Human Serum Albumin)	**Abbreviation**	**Target Receptor**
Lactose	Lac-HSA	Asialoglycoprotein Receptor
Mannose	Man-HSA	Mannose Receptor
Cyclic peptide C*SRNLIDC*	pPB-HSA	PDGFβ Receptor

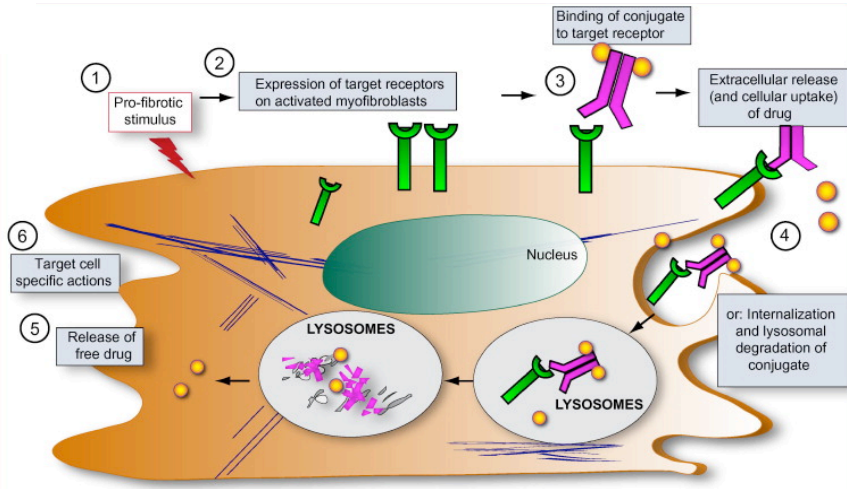

Figure 4; Drug targeting in the liver. Binding, uptake, internalization, and release of compounds targeted at extracellular receptors resulting in target cell-specific actions. *This figure is republished with permission*[73]

ALKALINE PHOSPHATASE

The liver serves as the main clearance mechanism for particles in the blood and therefore contains the majority of macrophages present in the body. In addition, the liver is the first-pass organ for gut derived toxins and foreign particles. It is becoming increasingly clear that these gut-derived products affect liver fibrogenesis, partly because the permeability of the intestinal wall changes during fibrogenesis[74-76]. Nowadays, the interplay between the intestine and the liver is receiving much attention, yet much is still unknown. In a healthy situation, small amounts of gut-derived lipopolysaccharide (LPS), a microbial compound and potent inducer of inflammation, are detoxified in the liver without significant inflammation. However, an increased influx of macrophages in the liver due to an inciting agent, combined with an increased permeability of the intestinal wall and a higher uptake of LPS, aggravates liver damage and fibrosis[75,76]. As shown in this thesis, macrophage activities regulate pro- and anti-fibrotic actions. LPS is known to induce a pro-inflammatory Th1 response and initiate M1 polarization, a process which is counterbalanced by accumulation of M2 wound healing macrophages[9,12]. So, persistent exposure of LPS to the liver may result in changes of the M1/M2 composition and subsequently chronic inflammation and fibrogenesis.

Liver fibrosis is associated with high serum alkaline phosphatase (AP) levels. Recent studies indicate that AP may act as a protective enzyme by dephosphorylation of LPS resulting in the non-toxic molecule Lipid A[77-80]. We hypothesized that high levels of AP may represent a physiological response to higher levels of this toxin during fibrosis. In **chapter 6** we describe our studies investigating the hepatic expression levels of AP in the acute and chronic phase of CCl_4-induced liver fibrosis. We studied the role of intestinal AP (iAP) on the progression of liver fibrosis, using iAP knock out mice (iAP KO). In addition, iAP was administered to fibrotic mice and we subsequently examined macrophage activity and fibrogenesis. Our data show that AP serves as a protective enzyme during the progression of liver fibrosis and changes in AP levels are associated with changes in the macrophage profile.

CONCLUSION

This study provides novel insights into the role of macrophages, and in particular the importance of the M1 and M2 composition during fibrogenesis. It is found that the composition of cells during the progression of disease changes and the concerted action of these different cell types and their mediators leads to homeostasis or, in case of chronic tissue damage, to a deregulated wound healing process. The local mediators PGE_2 and IFNγ fine-tune the complex cell-cell interactions in different phases of tissue damage and remodelling. Next to its effect on macrophages, PGE_2 is found to affect the HSC directly via Epac-1-dependant signalling mechanism. And finally, it is shown that alkaline phosphatase is an endogenous mediator during fibrosis, affecting fibroblast activation and fibrogenesis.

REFERENCES

1. Friedman SL. Hepatic fibrosis -- overview. *Toxicology*. 2008;254(3):120-129.

2. Schuppan D, Afdhal NH. Liver cirrhosis. *Lancet*. 2008;371(9615):838-851.

3. Duffield JS, Forbes SJ, Constandinou CM, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest*. 2005;115(1):56-65.

4. Fallowfield JA, Mizuno M, Kendall TJ, et al. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J Immunol*. 2007;178(8):5288-5295.

5. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003;3(1):23-35.

6. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958-969.

7. Laskin DL, Sunil VR, Gardner CR, Laskin JD. Macrophages and tissue injury: Agents of defense or destruction? *Annu Rev Pharmacol Toxicol*. 2011;51:267-288.

8. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004;25(12):677-686.

9. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol*. 2004;4(8):583-594.

10. Krausgruber T, Blazek K, Smallie T, et al. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol*. 2011;12(3):231-238.

11. Lekkerkerker AN, Aarbiou J, van Es T, Janssen RA. Cellular players in lung fibrosis. *Curr Pharm Des*. 2012;18(27):4093-4102.

12. Heymann F, Trautwein C, Tacke F. Monocytes and macrophages as cellular targets in liver fibrosis. *Inflamm Allergy Drug Targets*. 2009;8(4):307-318.

13. Beljaars L, Poelstra K, Molema G, Meijer DK. Targeting of sugar- and charge-modified albumins to fibrotic rat livers: The accessibility of hepatic cells after chronic bile duct ligation. *J Hepatol*. 1998;29(4):579-588.

14. Jansen RW, Molema G, Ching TL, et al. Hepatic endocytosis of various types of mannose-terminated albumins. what is important, sugar recognition, net charge, or the combination of these features. *J Biol Chem*. 1991;266(5):3343-3348.

15. Melgert BN, Olinga P, Van Der Laan JM, et al. Targeting dexamethasone to kupffer cells: Effects on liver inflammation and fibrosis in rats. *Hepatology*. 2001;34(4 Pt 1):719-728.

16. Cook PC, Jones LH, Jenkins SJ, Wynn TA, Allen JE, Macdonald AS. Alternatively activated dendritic cells regulate CD4+ T-cell polarization in vitro and in vivo. *Proc Natl Acad Sci U S A*. 2012;109(25):9977-9982.

17. Gordon S, Martinez FO. Alternative activation of macrophages: Mechanism and functions. *Immunity*. 2010;32(5):593-604.

18. Song E, Ouyang N, Horbelt M, Antus B, Wang M, Exton MS. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol*. 2000;204(1):19-28.

19. Sica A, Mantovani A. Macrophage plasticity and polarization: In vivo veritas. *J Clin Invest*. 2012;122(3):787-795.

20. Claassen MA, de Knegt RJ, Tilanus HW, Janssen HL, Boonstra A. Abundant numbers of regulatory T cells localize to the liver of chronic hepatitis C infected patients and limit the extent of fibrosis. *J Hepatol*. 2010;52(3):315-321.

21. Kanellakis P, Dinh TN, Agrotis A, Bobik A. CD4(+)CD25(+)Foxp3(+) regulatory T cells suppress cardiac fibrosis in the hypertensive heart. *J Hypertens*. 2011;29(9):1820-1828.

22. Kotsianidis I, Nakou E, Bouchliou I, et al. Global impairment of CD4+CD25+FOXP3+ regulatory T cells in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2009;179(12):1121-1130.

23. Kitani A, Fuss I, Nakamura K, Kumaki F, Usui T, Strober W. Transforming growth factor (TGF)-beta1-producing regulatory T cells induce smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF-beta1-mediated fibrosis. *J Exp Med*. 2003;198(8):1179-1188.

24. Liu F, Liu J, Weng D, et al. CD4+CD25+Foxp3+ regulatory T cells depletion may attenuate the development of silica-induced lung fibrosis in mice. *PLoS One*. 2010;5(11):e15404.

25. Louvet A, Teixeira-Clerc F, Chobert MN, et al. Cannabinoid CB2 receptors protect against alcoholic liver disease by regulating kupffer cell polarization in mice. *Hepatology*. 2011;54(4):1217-1226.

26. Galli SJ, Borregaard N, Wynn TA. Phenotypic and functional plasticity of cells of innate immunity: Macrophages, mast cells and neutrophils. *Nat Immunol*. 2011;12(11):1035-1044.

27. Calvaruso V, Maimone S, Gatt A, et al. Coagulation and fibrosis in chronic liver disease. *Gut*. 2008;57(12):1722-1727.

28. Marra F, Grandaliano G, Valente AJ, Abboud HE. Thrombin stimulates proliferation of liver fat-storing cells and expression of monocyte chemotactic protein-1: Potential role in liver injury. *Hepatology*. 1995;22(3):780-787.

29. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: Therapeutic translation for fibrotic disease. *Nat Med*. 2012;18(7):1028-1040.

30. Heymann F, Hammerich L, Storch D, et al. Hepatic macrophage migration and differentiation critical for liver fibrosis is mediated by the chemokine receptor C-C motif chemokine receptor 8 in mice. *Hepatology*. 2012;55(3):898-909.

31. Cataldo DD, Tournoy KG, Vermaelen K, et al. Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation. *Am J Pathol*. 2002;161(2):491-498.

32. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*. 2011;11(11):723-737.

33. Hirata T, Narumiya S. Prostanoids as regulators of innate and adaptive immunity. *Adv Immunol*. 2012;116:143-174.

34. Hirata T, Narumiya S. Prostanoid receptors. *Chem Rev*. 2011;111(10):6209-6230.

35. Hamberg M, Samuelsson B. On the metabolism of prostaglandins E 1 and E 2 in man. *J Biol Chem*. 1971;246(22):6713-6721.

36. Enomoto N, Ikejima K, Yamashina S, et al. Kupffer cell-derived prostaglandin E(2) is involved in alcohol-induced fat accumulation in rat liver. *Am J Physiol Gastrointest Liver Physiol*. 2000;279(1):G100-6.

37. Rincon-Sanchez AR, Covarrubias A, Rivas-Estilla AM, et al. PGE2 alleviates kidney and liver damage, decreases plasma renin activity and acute phase response in cirrhotic rats with acute liver damage. *Exp Toxicol Pathol*. 2005;56(4-5):291-303.

38. Ruwart MJ, Wilkinson KF, Rush BD, et al. The integrated value of serum procollagen III peptide over time predicts hepatic hydroxyproline content and stainable collagen in a model of dietary cirrhosis in the rat. *Hepatology*. 1989;10(5):801-806.

39. Mallat A, Gallois C, Tao J, et al. Platelet-derived growth factor-BB and thrombin generate positive and negative signals for human hepatic stellate cell proliferation. role of a prostaglandin/cyclic AMP pathway and cross-talk with endothelin receptors. *J Biol Chem*. 1998;273(42):27300-27305.

40. Haag S, Warnken M, Juergens UR, Racke K. Role of Epac1 in mediating anti-proliferative effects of prostanoid EP(2) receptors and cAMP in human lung fibroblasts. *Naunyn Schmiedebergs Arch Pharmacol*. 2008;378(6):617-630.

41. Weinberg E, Zeldich E, Weinreb MM, Moses O, Nemcovsky C, Weinreb M. Prostaglandin E2 inhibits the proliferation of human gingival fibroblasts via the EP2 receptor and epac. *J Cell Biochem*. 2009;108(1):207-215.

42. Huang S, Wettlaufer SH, Hogaboam C, Aronoff DM, Peters-Golden M. Prostaglandin E(2) inhibits collagen expression and proliferation in patient-derived normal lung fibroblasts via E prostanoid 2 receptor and cAMP signaling. *Am J Physiol Lung Cell Mol Physiol*. 2007;292(2):L405-13.

43. Kalinski P. Regulation of immune responses by prostaglandin E2. *J Immunol*. 2012;188(1):21-28.

44. Nataraj C, Thomas DW, Tilley SL, et al. Receptors for prostaglandin E(2) that regulate cellular immune responses in the mouse. *J Clin Invest*. 2001;108(8):1229-1235.

45. Tajima T, Murata T, Aritake K, et al. EP2 and EP4 receptors on muscularis resident macrophages mediate LPS-induced intestinal dysmotility via iNOS upregulation through cAMP/ERK signals. *Am J Physiol Gastrointest Liver Physiol*. 2012;302(5):G524-34.

46. Ratcliffe MJ, Walding A, Shelton PA, Flaherty A, Dougall IG. Activation of E-prostanoid4 and E-prostanoid2 receptors inhibits TNF-alpha release from human alveolar macrophages. *Eur Respir J*. 2007;29(5):986-994.

47. Zaslona Z, Serezani CH, Okunishi K, Aronoff DM, Peters-Golden M. Prostaglandin E2 restrains macrophage maturation via E prostanoid receptor 2/protein kinase A signaling. *Blood*. 2012;119(10):2358-2367.

48. Sheibanie AF, Khayrullina T, Safadi FF, Ganea D. Prostaglandin E2 exacerbates collagen-induced arthritis in mice through the inflammatory interleukin-23/interleukin-17 axis. *Arthritis Rheum*. 2007;56(8):2608-2619.

49. Sheibanie AF, Tadmori I, Jing H, Vassiliou E, Ganea D. Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. *FASEB J*. 2004;18(11):1318-1320.

50. Yao C, Sakata D, Esaki Y, et al. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat Med*. 2009;15(6):633-640.

51. Ganesh K, Das A, Dickerson R, et al. Prostaglandin E(2) induces oncostatin M expression in human chronic wound macrophages through axl receptor tyrosine kinase pathway. *J Immunol*. 2012;189(5):2563-2573.

52. Liu L, Ge D, Ma L, et al. Interleukin-17 and prostaglandin E2 are involved in formation of an M2 macrophage-dominant microenvironment in lung cancer. *J Thorac Oncol*. 2012;7(7):1091-1100.

53. Ylostalo JH, Bartosh TJ, Coble K, Prockop DJ. Human mesenchymal Stem/Stromal cells cultured as spheroids are self-activated to produce prostaglandin E2 that directs stimulated macrophages into an anti-inflammatory phenotype. *Stem Cells*. 2012;30(10):2283-2296.

54. Bansal R, Prakash J, Post E, Beljaars L, Schuppan D, Poelstra K. Novel engineered targeted interferon-gamma blocks hepatic fibrogenesis in mice. *Hepatology*. 2011;54(2):586-596.

55. de Rooij J, Zwartkruis FJ, Verheijen MH, et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*. 1998;396(6710):474-477.

56. Roscioni SS, Elzinga CR, Schmidt M. Epac: Effectors and biological functions. *Naunyn Schmiedebergs Arch Pharmacol*. 2008;377(4-6):345-357.

57. Grandoch M, Roscioni SS, Schmidt M. The role of epac proteins, novel cAMP mediators, in the regulation of immune, lung and neuronal function. *Br J Pharmacol*. 2010;159(2):265-284.

58. Dieter P, Altin JG, Decker K, Bygrave FL. Possible involvement of eicosanoids in the zymosan and arachidonic-acid-induced oxygen uptake, glycogenolysis and Ca2+ mobilization in the perfused rat liver. *Eur J Biochem*. 1987;165(2):455-460.

59. Okumura T, Kanemaki T, Kitade H. Stimulation of glucose incorporation into glycogen by E-series prostaglandins in cultured rat hepatocytes. *Biochim Biophys Acta*. 1993;1176(1-2):137-142.

60. Treffkorn L, Scheibe R, Maruyama T, Dieter P. PGE2 exerts its effect on the LPS-induced release of TNF-alpha, ET-1, IL-1alpha, IL-6 and IL-10 via the EP2 and EP4 receptor in rat liver macrophages. *Prostaglandins Other Lipid Mediat*. 2004;74(1-4):113-123.

61. Dieter P, Hempel U, Kamionka S, et al. Prostaglandin E2 affects differently the release of inflammatory mediators from resident macrophages by LPS and muramyl tripeptides. *Mediators Inflamm*. 1999;8(6):295-303.

62. Hui AY, Cheng AS, Chan HL, et al. Effect of prostaglandin E2 and prostaglandin I2 on PDGF-induced proliferation of LI90, a human hepatic stellate cell line. *Prostaglandins Leukot Essent Fatty Acids*. 2004;71(5):329-333.

63. Hui AY, Dannenberg AJ, Sung JJ, et al. Prostaglandin E2 inhibits transforming growth factor beta 1-mediated induction of collagen alpha 1(I) in hepatic stellate cells. *J Hepatol*. 2004;41(2):251-258.

64. Huang SK, Wettlaufer SH, Chung J, Peters-Golden M. Prostaglandin E2 inhibits specific lung fibroblast functions via selective actions of PKA and epac-1. *Am J Respir Cell Mol Biol*. 2008;39(4):482-489.

65. Kojima F, Kapoor M, Kawai S, Yang L, Aronoff DM, Crofford LJ. Prostaglandin E2 activates Rap1 via EP2/EP4 receptors and cAMP-signaling in rheumatoid synovial fibroblasts: Involvement of Epac1 and PKA. *Prostaglandins Other Lipid Mediat*. 2009;89(1-2):26-33.

66. Refsnes M, Thoresen GH, Dajani OF, Christoffersen T. Stimulation of hepatocyte DNA synthesis by prostaglandin E2 and prostaglandin F2 alpha: Additivity with the effect of norepinephrine, and synergism with epidermal growth factor. *J Cell Physiol*. 1994;159(1):35-40.

67. Kimura M, Osumi S, Ogihara M. Prostaglandin E(2) (EP(1)) receptor agonist-induced DNA synthesis and proliferation in primary cultures of adult rat

hepatocytes: The involvement of TGF-alpha. *Endocrinology*. 2001;142(10):4428-4440.

68. Poelstra K, Prakash J, Beljaars L. Drug targeting to the diseased liver. *J Control Release*. 2012;161(2):188-197.

69. Borkham-Kamphorst E, Kovalenko E, van Roeyen CR, et al. Platelet-derived growth factor isoform expression in carbon tetrachloride-induced chronic liver injury. *Lab Invest*. 2008;88(10):1090-1100.

70. Wong L, Yamasaki G, Johnson RJ, Friedman SL. Induction of beta-platelet-derived growth factor receptor in rat hepatic lipocytes during cellular activation in vivo and in culture. *J Clin Invest*. 1994;94(4):1563-1569.

71. Beljaars L, Weert B, Geerts A, Meijer DK, Poelstra K. The preferential homing of a platelet derived growth factor receptor-recognizing macromolecule to fibroblast-like cells in fibrotic tissue. *Biochem Pharmacol*. 2003;66(7):1307-1317.

72. Hagens WI, Mattos A, Greupink R, et al. Targeting 15d-prostaglandin J2 to hepatic stellate cells: Two options evaluated. *Pharm Res*. 2007;24(3):566-574.

73. Poelstra K, Schuppan D. Targeted therapy of liver fibrosis/cirrhosis and its complications. *J Hepatol*. 2011;55(3):726-728.

74. Enomoto N, Ikejima K, Bradford BU, et al. Role of kupffer cells and gut-derived endotoxins in alcoholic liver injury. *J Gastroenterol Hepatol*. 2000;15 Suppl:D20-5.

75. Enomoto N, Ikejima K, Yamashina S, et al. Kupffer cell sensitization by alcohol involves increased permeability to gut-derived endotoxin. *Alcohol Clin Exp Res*. 2001;25(6 Suppl):51S-4S.

76. Pradere JP, Troeger JS, Dapito DH, Mencin AA, Schwabe RF. Toll-like receptor 4 and hepatic fibrogenesis. *Semin Liver Dis*. 2010;30(3):232-244.

77. Poelstra K, Bakker WW, Klok PA, Hardonk MJ, Meijer DK. A physiologic function for alkaline phosphatase: Endotoxin detoxification. *Lab Invest*. 1997;76(3):319-327.

78. Poelstra K, Bakker WW, Klok PA, Kamps JA, Hardonk MJ, Meijer DK. Dephosphorylation of endotoxin by alkaline phosphatase in vivo. *Am J Pathol*. 1997;151(4):1163-1169.

79. Tuin A, Huizinga-Van der Vlag A, van Loenen-Weemaes AM, Meijer DK, Poelstra K. On the role and fate of LPS-dephosphorylating activity in the rat liver. *Am J Physiol Gastrointest Liver Physiol*. 2006;290(2):G377-85.

80. Koyama I, Matsunaga T, Harada T, Hokari S, Komoda T. Alkaline phosphatases reduce toxicity of lipopolysaccharides in vivo and in vitro through dephosphorylation. *Clin Biochem*. 2002;35(6):455-461.

CHAPTER 2

Hepatic localisation of M1 and M2 macrophages during fibrogenesis and resolution of fibrosis in mice and man

Leonie Beljaars[1], Marlies Schippers[1], Catharina Reker-Smit[1], Fernando Martinez[2], Laura Helming[3], Klaas Poelstra[1], Barbro N. Melgert[1]

[1] Dept. of Pharmacokinetics, Toxicology and Targeting, Groningen, The Netherlands
[2] Kennedy Institute of Rheumatology and Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, UK
[3] Institute for Medical Microbiology, Immunology and Hygiene, Technische Universität München, Munich, Germany

ABSTRACT

Macrophages can display several, even opposing activities during fibrosis. Currently, it is recognized that different phenotypes of activated macrophages may both promote fibrosis or contribute to its resolution. The best-characterized phenotypes are labeled M1 (classically activated) and M2 (alternatively activated). The *in vivo* appearance of these macrophage phenotypes, their localization and activities during fibrogenesis and resolution of fibrosis are poorly characterized. In this study, we therefore aimed to localize and quantify M1 and M2 macrophages in diseased mouse and human livers.

The scarred collagen-rich areas in end-stage cirrhotic human livers and in CCl_4-damaged mouse livers contained many macrophages and even giant CD68+ cells. Of note, the number of macrophages residing in the parenchymal areas of fibrotic livers was significantly reduced compared to normal. The scar-associated macrophages were further characterized as either M1 (IRF5) or M2 (CD206, TGM2 and YM1) and higher numbers of these M1 and M2 cells were detected in diseased livers as compared to normal. Interestingly, in mouse livers undergoing resolution of fibrosis, the total number of macrophages was significantly lower compared to their fibrotic counterparts. Remarkably, M1 (IRF5+) numbers were not lower in livers undergoing resolution, but M2 (YM1+) macrophages were almost completely gone.

In conclusion, this study shows the presence of M1 and M2 macrophages side by side in fibrotic lesions in human and mouse livers, indicating that M1 and M2 phenotypes are both necessary in the fibrotic response. During resolution of fibrosis, M1 macrophages remain, whereas M2 are almost absent. A balance of M1 versus M2 macrophages may play a key role in fibrogenesis and resolution, and manipulation of this balance may be of therapeutic use.

INTRODUCTION

Chronic injury of the liver leads to the induction of fibrogenic processes that ultimately can progress to cirrhosis, a state in which excessive extracellular matrix deposition hampers normal liver functions. Hepatic Stellate Cells (HSC) are regarded as the principal cells that are involved in scar tissue deposition[1,2]. Recent studies indicate that the role of Kupffer cells has been underestimated in fibrogenesis and consequently hepatic macrophages have gained more attention recently[3-5]. Kupffer cells are well-known producers of reactive oxygen species, cytokines and chemokines, that perpetuate hepatic inflammatory responses, and of matrix-degrading enzymes. In addition, these macrophages can phagocytose microorganisms, apoptotic cells and cellular debris generated during tissue injury and remodeling. Duffield *et al*[6] clearly showed that Kupffer cells can exert different, even opposing roles during various stages of liver fibrosis. They showed that macrophage activities during the injury phase were predominantly associated with promotion of matrix deposition and HSC activities, while during recovery macrophages were associated with enhanced resolution and higher production of matrix metalloproteinases (MMP)[6,7]. These diverse roles may indicate that activated macrophages differentiate into diverse phenotypes during various stages of liver disease.

Activated macrophages are described to polarize into different phenotypes depending on signals they receive from their environment. Many types can be distinguished, but the best-characterized are classically activated macrophages (also called M1) and alternatively activated macrophages (also called M2)[8-11]. In general, M1 macrophages are activated by interferon-gamma and TNFα (or endotoxin leading to TNFα release) leading to enhanced microbicidal and tumoricidal capacity and secretion of high levels of pro-inflammatory cytokines and chemokines. M1 macrophages can also inhibit fibrotic activities of fibroblasts by releasing antifibrogenic or fibrolytic factors such as MMPs[12-14]. M2 macrophages, activated by interleukin-4 and interleukin-13, are associated with increased fibrogenesis, tissue remodelling, and angiogenesis[13,15,16]. *In vitro*, Song et al[13] showed that the M2 macrophages produced the profibrogenic factors PDGFBB and TGFbeta and that these M2 activated cells increased collagen production and proliferation of fibroblasts. Although M2 macrophages are predominantly considered to be pro-fibrotic, they are also associated with anti-fibrotic properties, which may be explained by the different M2 phenotypes that exist[5,9]. For instance, M2 macrophages can phagocytose apoptotic cells and matrix components via mannose and scavenger receptors[17-19]. In addition, Pesce et al[20] showed that arginase-1 expressing M2 cells were related to suppression rather than induction of fibrosis.

Different markers were identified *in vitro* to distinguish M1 and M2 macrophages[9]. Interferon-regulatory factor-5 (IRF-5) was recently described as a marker that is specifically expressed in M1 macrophages[21]. Polarization to M2 can be detected by increased upregulation of the mannose receptor (MRC1; also indicated as CD206, mouse and human) or chitinase-like secretory protein YM1 (mouse)[22-25]. Recently, transglutaminase-2 (TGM2) was identified as a new human and murine M2 marker[26]. So far, most of the knowledge generated about the different macrophage subsets is derived from *in vitro* studies. How these concepts fit *in vivo*, in particular in liver diseases, is largely unexplored.

In vivo localization of various macrophage subtypes has not been well described for liver diseases, although the importance of macrophage polarization is increasingly recognized in liver diseases[4,5,7,27,28]. Therefore, we studied M1 and M2 macrophages in diseased mouse and human livers using immunohis-

tochemical techniques and western blot analysis with the aim to relate their localization and numbers *in vivo* to previously described biological functions.

MATERIAL & METHODS

Animals

Male mice (BALB/c, ±25 mg) were obtained from Harlan (Zeist, The Netherlands) and housed in a temperature-controlled room with 12 hr light/dark regimen. The animal experiments were approved by the Local Committee on Animal Experimentation and were performed according to strict governmental and international guidelines on animal experimentation.

Animal models

Acute liver injury model. Mice were sacrificed 72 hours after a single intraperitoneal injection of CCl_4 (1 ml/kg CCl_4 diluted in olive oil).

Chronic liver injury (fibrosis) model. Mice received twice-weekly intraperitoneal injections of CCl_4 for 4 or 8 weeks. The dose of CCl_4 was gradually increased (diluted in olive oil; week 1: 0.5 ml/kg, week 2: 0.8 ml/kg, week 3-8: 1 ml/kg). Mice were sacrificed after 4 or 8 weeks reflecting early and advanced fibrosis respectively.

Resolution model: Mice received CCl_4 for 4 weeks (with increasing CCl_4 doses as described in the previous section. After 4 weeks, CCl_4 administration was stopped and the mice were allowed to recover for a week after which they were sacrificed (n=6 per group).

Human Livers

Residual human liver tissue was obtained from the Department of Hepato-Pancreato-Biliary Surgery and Liver Transplantation (University Medical Center Groningen (UMCG), the Netherlands) according to the Dutch Code of Conduct (http://www.federa.org/gedragscodes-codes-conduct-en) and the UMCG Research Code (http://www.rug.nl/umcg/onderzoek/researchcode/index?lang=en). Normal human liver tissue (n=10) was obtained from residual liver tissue from patients undergoing partial hepatectomy because of metastasis of colorectal carcinoma and from donor livers discarded for transplantation because of technical reasons. Cirrhotic human liver tissue (n=6) was obtained from patients undergoing liver transplantation. Indications for transplantation were primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC), congenital cirrhosis, and Wilson's cirrhosis.

*Tissue processing: T*issue specimens from at least 3 different mouse liver lobes were snap frozen in isopentane (-80°C) for immunohistochemical analysis, or in N_2(l) for Western blot analysis. A wedge (10–60 g) of freshly obtained human livers was cut, perfused with cold University of Wisconsin organ storage solution (DuPont Critical Care, Waukegan, IL) immediately after resection, and pieces were snap-frozen in isopentane (-80°C).

Immunohistochemical analysis: Acetone-fixed cryostat sections (4 μm) were stained according to standard immunohistochemical procedures with 3-amino-9-ethyl-carbazole in the final step (32). Sec-

tions were incubated with the primary antibody for 1h. Primary antibodies to detect fibrotic extracellular matrix (polyclonal goat anti-collagen type I from Southern Biotech), macrophages (mouse anti-human CD68 (DAKO), monoclonal rat anti-mouse CD68 (AbD Serotec, Düsseldorf, Germany), and polyclonal rabbit anti-human CD68 (Santa Cruz Biotechnology), M1 macrophages (polyclonal rabbit anti-human and mouse IRF5; Protein Tech, Manchester, UK), and M2 macrophages (polyclonal goat anti-mouse chitinase 3-like/ECF-L (YM1; R&D), rabbit anti-human TGM2 (AbD Serotec) and CD206 (rat anti-mouse CD206 and mouse anti-human CD206 both from BioLegends (ITK Diagnostics, Uithoorn, The Netherlands) were used. Staining of CD68 was quantified by image analysis with Cell^D analysis program (Olympus, Zoetermeer, The Netherlands).

To detect co-localization, we used double-staining techniques with peroxidase&AEC (red) and alkaline phosphatase&Naphtol AS-MX phosphate/Fast Blue BB (blue) [29]. Double stainings containing IRF-5 and CD206 were visualized with NovaRed (red) and BCIP/NBT (blue) from Vector Laboratories.

Western Blot analysis: Tissues samples were homogenized on ice in cold RIPA buffer (50mM Tris-HCl, 150mM NaCl, 0.1% SDS, 0.1% Igepal in 0.5% sodium deoxycholate with 1 tablet of protease inhibitor cocktail and 1 tablet of phosphatase inhibitor (Roche Diagnostics, Mannheim, Germany)) and the lysates were centrifuged for 1h (13,000 rpm, 4°C). The supernatants were stored at -80°C. Total protein (100 µg) from each sample was applied on SDS-PAGE (10%), transferred to polyvinylidene difluoride membranes, and incubated overnight at 4°C with the indicated primary antibodies. After washing and incubation with secondary horseradish peroxidase-coupled antibodies, the protein bands were visualised with ECL (Perkin-Elmer, Groningen, The Netherlands) and quantified by G-Box (Syngene, Cambridge, UK).

Statistical analysis

Results are expressed as means ±SEM. Data were analyzed using Student's t tests with Welsh corrections (Graph Pad software) and differences were considered significant $p<0.05$.

RESULTS

Localisation of CD68 in human and mouse fibrotic livers

After acute and chronic CCl_4 damage, pericentral necrosis led to a wound-healing response with influx of myofibroblasts and increased collagen deposition (fig.1a,b) in mouse livers. As compared to normal livers, a higher number of CD68-positive cells was found in fibrotic livers, and these macrophages predominantly concentrated in necrotic areas after acute injury and in scars during advanced fibrosis (fig. 1.c,d). Additionally, the appearance of several scar-associated macrophages was changed as compared to macrophages in normal livers and some of these cells resembled giant cells (fig.1c, indicated by arrowheads in insert). Remarkably, significantly less staining for CD68+ cells was found in the parenchymal areas of fibrotic livers as compared to normal (fig.1e,f).

In human cirrhotic livers, a similar distribution of macrophages was found (fig.2). Collagen deposition was greatly increased in these end-stage cirrhotic livers (fig.2a,b). Macrophages (CD68+) were prominently present in cirrhotic scars irrespective of the origin of cirrhosis (PBC, PSC, congenital cirrhosis, and Wil-

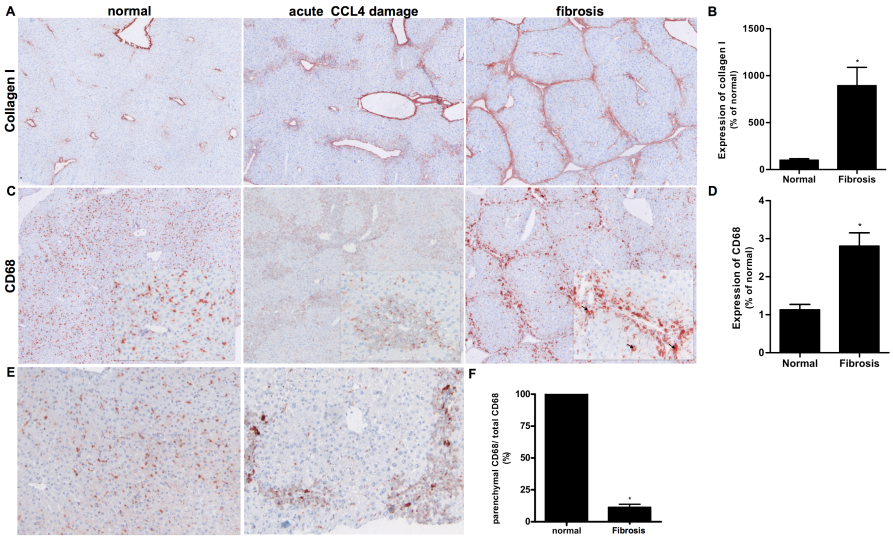

Figure 1. Expression and localization of macrophages in normal livers and in livers after acute and chronic CCL4 damage. (**A**) Immunohistochemical and (**B**) Western blot analysis of extracellular matrix deposition (collagen type I). (**C**) Immunohistochemical localization of macrophages (CD68). Note the increased size of certain CD68-positive macrophages in fibrotic livers (arrow heads in insert C). (**D**) Image analysis of total CD68 staining in mice livers. (**E-F)** CD68 staining and quantification in the parenchymal areas of normal and fibrotic livers. While the total area of CD68+ cells was increased in fibrotic livers, a significantly lower CD68-stained area was found in the parenchyma of fibrotic livers as compared to normal. Magnifications 4x (**A,C**), 10x (**E**) and 20x (inserts). N=6/group.

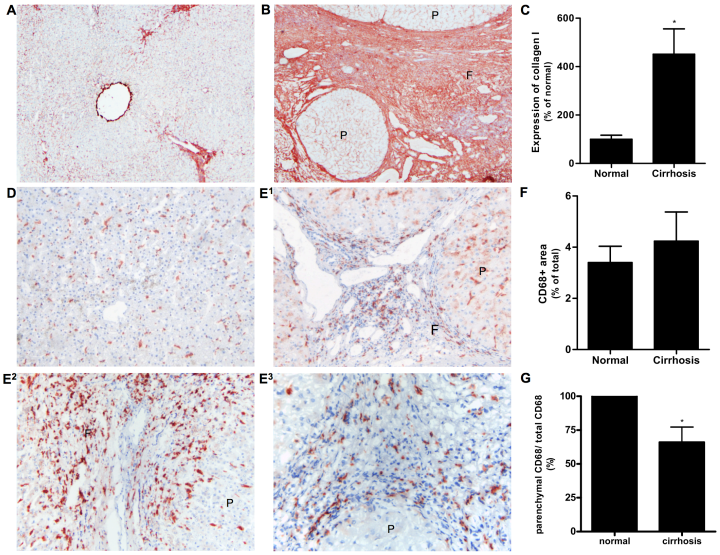

Figure 2. Expression and localization of macrophages in normal and cirrhotic human livers. (**A,B**) Immunohistochemical and (**C**) Western blot analysis of extracellular matrix deposition (collagen type I). Immunohistochemical localization of macrophages (CD68) in (**D**) normal and (**E**) cirrhotic livers. In cirrhotic livers of various origins (**E^1**. PBC, **E^2**. PSC, **E^3**. congenital cirrhosis), the presence of macrophages was detected in the collagenous fibrotic bands. (**F-G**) Image analysis of CD68 staining in human livers. A significantly reduced CD68 staining was found in the parenchymal area of human cirrhotic livers as compared to normal (**G**). Magnifications: 4x (**A,B**), 10x (**D,E^1,E^2**) and 20x (**E^3**). f=fibrotic matrix, p=liver parenchyma. N>5/group.

son's disease) (fig.2d,e). The total number of CD68+ cells was somewhat, though not significantly, higher in cirrhotic livers as compared to normal (fig.2f). Again, less staining for CD68 was found in the parenchymal areas of cirrhotic livers as compared to healthy livers (fig.2g).

M1 macrophages in mouse and human livers

IRF-5 was used to identify M1 macrophages in mouse and human livers. IRF-5 staining completely co-localized with CD68 in both mouse and human livers (fig.3a). It was clear that only a subset of the total number of macrophages expressed IRF5. To prove the phenotype-specificity of this M1 marker, we performed double-immunostainings of IRF-5 and the M2 marker CD206 in human livers and found no co-localization (fig.3b). Microscopic analysis showed that IRF-5 staining in mouse livers was present in cells within necrotic areas after acute damage and in cells in scars during advanced fibrosis (fig.3c). The staining of IRF-5 in human livers was also predominantly present the septa (fig.3e). Western blot analysis of liver homogenates revealed a significantly higher expression of IRF-5 in diseased mouse and human livers as compared to healthy livers (fig.3d,f).

M2 macrophages in mouse and human livers

Subsequently, we studied the hepatic distribution of M2 macrophages (fig.4). CD206 is a well-known marker for both mouse and human M2 macrophages. CD206/CD68 double-positive cells were present in fibrotic livers and were predominantly found in the scars (fig.4a). In addition to this, CD206 staining was present in liver parenchyma and this staining most likely reflected expression of CD206 on sinusoidal endothelial cells (co-localisation with CD31). The pronounced endothelial staining of CD206 complicates interpretation of Western blot quantification of CD206. Microscopic evaluation of sections double

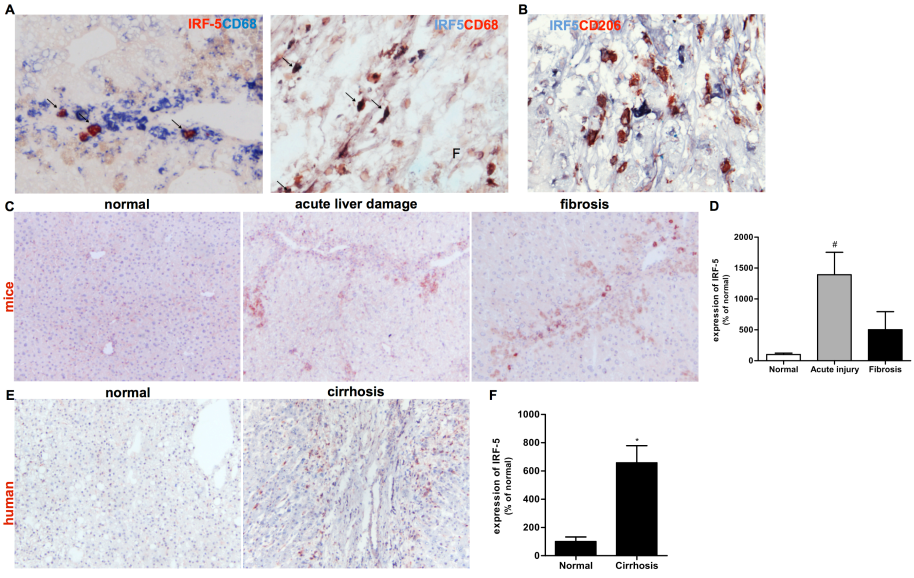

Figure 3. Localisation and quantification of IRF-5 (M1) in mouse and human livers. (**A**) Colocalisation of IRF-5 and CD68 in mouse and human livers. (**B**) Double-staining for IRF5 (blue staining) and (CD206 (red staining) showed no colocalisation. (**C,D**) Immunohistochemical and Western blot analysis of IRF-5 in normal mice livers and in livers after acute and chronic CCl4 damage. (**E,F**) IRF5 staining and Western blot analysis of normal and cirrhotic human livers. Magnifications: 10x (**C,E**), 40x (**A,B**), (*p<0.05). N>5/group.

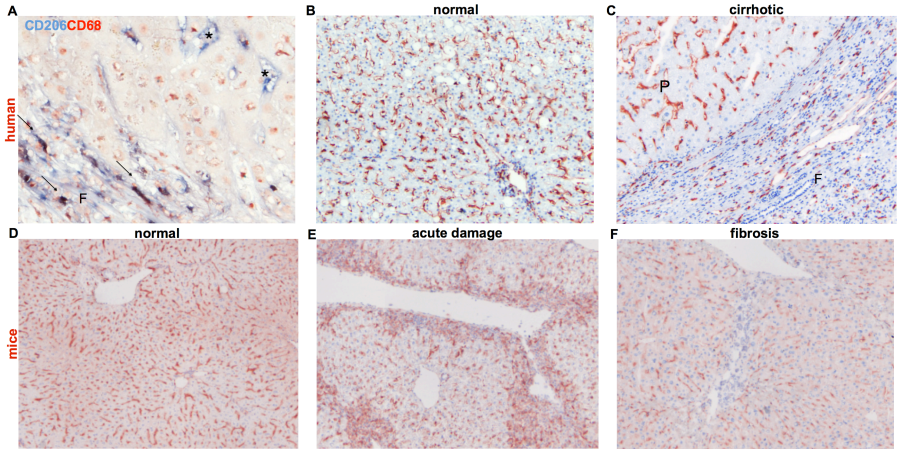

Figure 4. Immunohistochemical staining for CD206 (MCR-1; mannose receptor) in (A-C) human and (D-F) mouse livers. (**A**) Colocalisation of CD206 (blue staining) and CD68 (red staining). Arrows indicate colocalization, asterisks indicate endothelial staining of CD206. Magnifications: 10x (**B-F**), 40x (A). N>5/group.

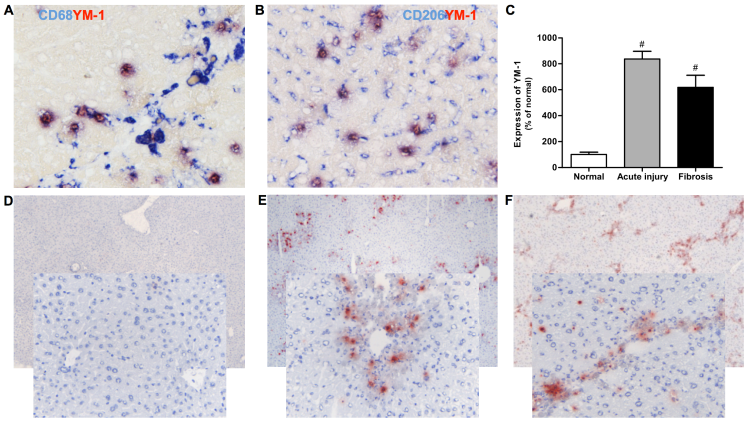

Figure 5. Localisation and quantification of YM1 (M2) in mouse livers. (**A**) Colocalisation of YM1 (red staining) and CD68 (blue staining). (**B**) Colocalisation of YM1 (red staining) and CD206 (blue staining). (**C**) Western blot analysis of YM1 in liver homogenates indicated significantly higher expression of YM1 in diseased mouse livers. (**D-F**). Immunohistochemical localisation of YM1 in livers of (**D**) normal mice, (**E**) after acute CCl_4-injury and (**F**) in advanced fibrosis . Magnifications 4x (**D-F**), 20x (inserts), 40x (**A,B**). *p<0.05. N=6/group.

stained for CD206 and CD68 indicated that double positive cells were more frequent in fibrotic liver than in normal livers, but Western blot analysis of the same livers showed unchanged or even reduced expression in respectively human and mouse livers (data not shown).

YM1 was used as an additional M2 marker for mouse livers [22]. Expression of YM-1 is restricted to mice and we could therefore not use it for human liver tissue. YM1 co-localized with CD68 and with CD206 (fig.5a,b). All cells that expressed YM1 were positive for CD68 and CD206, but not all CD68-positive cells stained positive for YM1. As compared to normal mouse livers, the expression of YM1 was clearly higher

after acute and chronic CCl_4 damage as demonstrated by western blot analysis (fig.5c) and immunohistochemical staining (fig.5d-f). In diseased mouse livers, a clear staining of YM1 in necrotic and fibrotic areas was found.

The recently described M2 marker TGM2[26] was also used to identify M2 macrophages in human livers (fig.6). Immunohistochemical staining for TGM2 resulted in staining of the parenchymal area of the livers, mostly staining hepatocytes, but in cirrhotic livers additional strong positive cells were found in septa (fig.6a,b). TGM2 staining present in scars co-localized with CD68 (fig.6c) and with CD206 (fig.6d) confirming presence of TGM2 in hepatic M2 macrophages that accumulate in these areas. Quantitative evaluation is difficult for this marker as well, because hepatocytes are positive for TGM2 too and therefore no significant differences could be detected between normal and cirrhotic livers (data not shown).

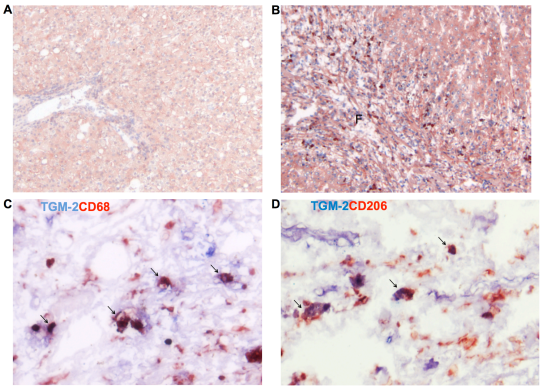

Figure 6. Localisation and quantification TGM2 (M2) in human livers. Immunohistochemical localisation of TGM2 in (**A**) normal and (**B**) cirrhotic human livers. Note the presence of the strongly stained cells in the fibrotic matrix (F). (**C**) Colocalisation of TGM2 (blue staining) and CD68 (red staining). (**D**) Colocalization of TGM2 (blue staining) and CD206 (red staining). Arrows indicate colocalisation. Magnifications: 10x (**A-B**), 40x (**C-D**). N>5/group.

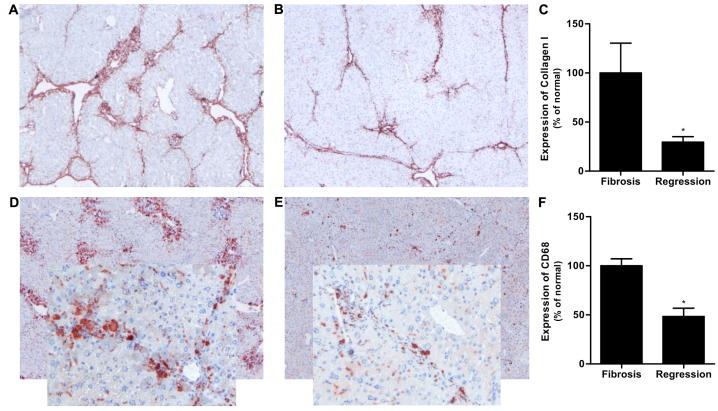

Figure 7. Immunohistochemical and Western blot analysis of the hepatic expressions of (A-C) collagen type I and (D-F) macrophages (CD68) in fibrosis (4 weeks of CCl4 in mice (**A,D**) **and in livers undergoing resolution** (after cessation of CCl_4 administration (**B,E**). * p<0.05. Magnifications 4x; insert 20x. N=6/group.

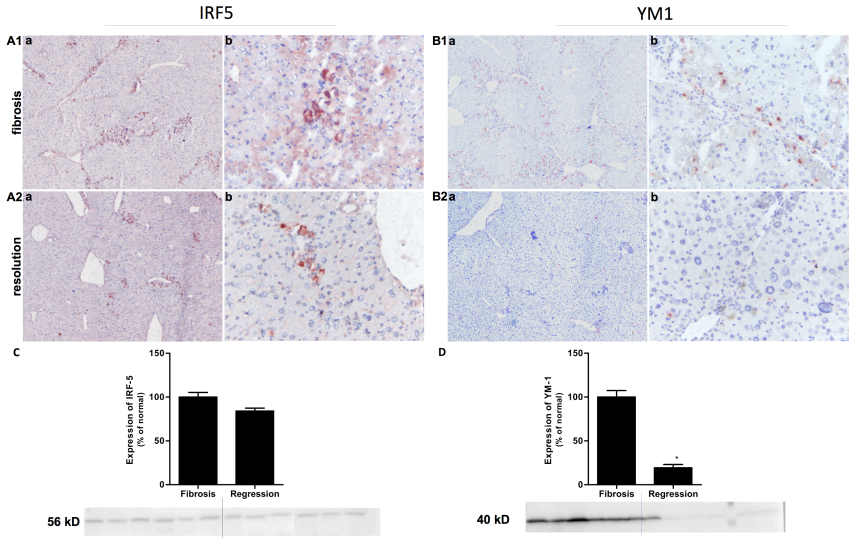

Figure 8. Expressions of IRF5 (M1; figs A) and YM1 (M2; figs B) in fibrotic mouse livers (4 weeks CCl_4, figs 1/ upper panel) **and in fibrotic livers undergoing resolution** (after cessation of CCl_4 administration; figs 2/ lower panel). Figures demonstrate an overview (**A**) and close up (**B**; magnification 20x). (**C**) Western blot quantification of IRF5 expression in fibrosis versus resolution, and (**D**) Western blot quantification of YM1 expression in fibrosis versus resolution. *p<0.05. N=6/group.

M1 and M2 macrophages in a mouse model of resolution

Cessation of fibrosis-inducing agents induces reversal of the fibrotic process[30]. This is also apparent in our mouse model with lower hepatic collagen type I in livers of mice in which CCl_4 administration was stopped versus their fibrotic equivalents (fig.7a-c). Since macrophages are important during resolution[6,7], we studied the localisation and quantity of macrophage phenotypes in these 2 groups of mice. Expression of CD68 was significantly lower in livers undergoing resolution as compared to their fibrotic counterparts (fig.7d-f). When we examined the activation of macrophages in these two groups, no significant differences were found in the expression of IRF5 (fig.8a+c). However, a clear difference in the number of M2 macrophages was found (fig.8b+d). YM1 staining was abundantly present in fibrotic livers, but in livers undergoing resolution this M2 marker was almost completely gone (fig.8b). Western blot analysis revealed a reduction of 81±8% in YM1 expression during resolution.

DISCUSSION

Increasing awareness of the diverse activities of macrophage phenotypes is leading to improved understanding of the role of macrophages in fibrotic processes. Knowledge about the different macrophage phenotypes has its roots in *in vitro* studies. These *in vitro* studies have been essential to discover markers to distinguish M1 and M2 macrophages and to identify the specific activities of these subsets. How these *in vitro*-generated phenotypes relate to macrophages *in vivo* is largely unknown. Moreover, *in vivo* localizations and biological roles of these phenotypes are relatively unexplored in liver diseases. In this study, we aimed to identify macrophage subsets in human and mouse livers and the changes that occur during

disease. We showed that M1 and M2 macrophages were simultaneously present in the scars of human and mice cirrhotic livers. Furthermore, we found that M2 macrophages disappeared in fibrotic livers undergoing resolution, while M1 macrophages persisted.

Krausgruber *et al*[21] showed high expression of IRF-5 in human M1 macrophages in culture, while M2 and non-activated macrophages did not express IRF5. We now show that IRF-5 can be used to identify a subset of macrophages *in vivo* in human and mouse livers. Our study clearly demonstrates that M1 macrophages (CD68/IRF5+ cells) are significantly increased in diseased livers as compared to normal. IRF5+ cells are located in necrotic areas in acutely damaged livers and in fibrous septa in advanced fibrosis. These localizations might correspond to reported *in vitro* M1 activities such as production of pro-inflammatory cytokines and chemokines[5,9,13]. The observation that M1 macrophages are still present in livers undergoing resolution might be related to their ability to produce MMPs[10,12-14,31]. Classical activation of macrophages *in vitro* resulted in induced expression of for instance MMP7 and MMP9 and both may be necessary in the collagenous scars for removal of collagen fibers. In addition, Fallowfield *et al*[7] demonstrated increased hepatic MMP13 expression by scar-associated macrophages in CCl_4-damaged livers and it was found that resolution of CCl_4-induced fibrosis was retarded in MMP13-deficient mice. However, macrophage phenotypes in these scars were not further characterized. We now show with our localization studies that during fibrogenesis scar-associated macrophages are both of M1 and M2 phenotype, while during resolution the scar-associated macrophages are predominantly M1 cells. It therefore appears that M1 macrophages may be responsible for the MMP13 production that is necessary for resolution. Colocalisation studies with IRF-5 and MMP13 may provide additional insights.

To identify M2 macrophages we started with the well-known marker CD206 (mannose receptor, MCR1)[9]. While in many organs M2 macrophages specifically express CD206, in livers CD206 expression is found in macrophages as well as in sinusoidal endothelial cells. This makes quantitative interpretations for instance by western blot or PCR difficult. An option would be to microscopically count CD206+CD68+ macrophages[32]. However, this is error-prone and dependent on the quality of the double-staining and how the marker is expressed. Also, quantitative double-stainings are not always possible for every marker. Our studies show that YM1 is a selective marker for M2 macrophages in mouse livers and can be used to quantify M2 expression. However, this marker is only present in rodents[22] and cannot be used for human tissues. Transglutaminase-2 is a novel marker for M2 macrophages recently described in lungs[26]. The advantage of TGM2 is that this marker is conserved in mice and humans. We now show that TGM2 is co-expressed in CD68+ and in CD206+ cells in fibrotic septa in human and mouse livers, confirming its presence in M2 hepatic macrophages. Although the hepatic expression is not limited to macrophages, as can be seen in figure 6, TGM2 staining in the scar-associated macrophages in cirrhotic livers is much stronger than in other hepatic cells. TGM2 is a multifunctional enzyme involved in transamidation and cross-linking of proteins. It is also linked to apoptosis, cellular differentiation and matrix stabilization[33-35]. In liver, Popov *et al*[36] showed that TGM2 is enhanced in mice with CCl_4-induced fibrosis, but they found no relationship between TGM2 and stabilization of fibrotic matrix. However, TGM-2 expression was not related to macrophage activities. To summarize, using a combination of the markers CD206, YM1 and TGM2 we are able to show that M2 macrophages are present in scar tissue during hepatic fibrogenesis.

This study clearly shows the presence of M1 and M2 macrophages side by side in fibrotic lesions in human and mouse livers, indicating that apparently M1 and M2 phenotypes are both necessary in the fi-

brotic response. The question remains how these macrophage phenotypes interact with each other and with other resident cells to enhance or dissolve fibrosis. Song et al[13] for instance showed that M2 macrophages increased the proliferation index and collagen synthesis of co-cultivated WI-38 fibroblasts, while M1 macrophages markedly reduced collagen production by these cells. Most *in vitro* studies suggest that M2 activation results in enhanced fibrogenesis, while M1 macrophages inhibit fibrogenesis by releasing antifibrogenic or fibrolytic factors. Just recently, Lopez Navarrette[15] showed the importance of M2 macrophages in fibrogenesis in a CCl_4-induced model of liver fibrosis in which Kupffer cells were stimulated to polarize to an M2 phenotype after hepatic inoculation of Taenia Crassiceps larvae. Our results also suggest a more profibrotic character of M2 macrophages, because M2 markers were present in fibrotic lesions in human and mouse livers, but were nearly absent in these same lesions during resolution of fibrosis. The disappearance of M2 macrophages during resolution indicates that M1 activities may be more important in this phase. M1 macrophages have been reported to be major producers of various MMPs and MMP-producing macrophages were previously reported to be present during liver regeneration in mice[3,7,12]. However, M2 macrophages were found to be important cells for efferocytosis and phagocytosis of matrix debris[14,18,37-39], and these characteristics of M2 cells may also be necessary during the resolution phase. The reason we do not see M2 macrophages anymore in our resolution model, may be caused by the fact that the resolution is already nearing its end and these functions of M2 macrophages may have become redundant.

In conclusion, using a set of well-established as well as recently identified markers we now clearly show local accumulation of both M1 and M2 macrophages in fibrotic septa of mouse and human end-stage cirrhotic livers. This provides a basis for further exploring the different activities of these macrophage phenotypes during liver fibrosis and resolution of fibrosis. The observation that during liver remodeling M1 macrophages persist and M2 macrophages disappear indicates that different combinations of M1 versus M2 macrophages may play a key role in fibrogenesis and resolution. Manipulation of their combinations may therefore be of therapeutic value.

ACKNOWLEDGEMENTS

We gratefully acknowledge the surgeons of the Department of Hepato-Pancreato-Biliary Surgery and Liver Transplantation (University Medical Center Groningen, Groningen, The Netherlands) for providing us with healthy and cirrhotic human liver tissue. In addition, we thank students Pharmacy (Jasper Lodder, Karyn Feenstra, Elze Alons and Nour Aughsteen) for their practical contributions to this paper.

REFERENCES

1. Pinzani M, Rombouts K, Colagrande S. Fibrosis in chronic liver diseases: Diagnosis and management. *J Hepatol*. 2005;42 Suppl(1):S22-36.
2. Friedman SL. Evolving challenges in hepatic fibrosis. *Nat Rev Gastroenterol Hepatol*. 2010;7(8):425-436.
3. Ramachandran P, Iredale JP. Macrophages: Central regulators of hepatic fibrogenesis and fibrosis resolution. *J Hepatol*. 2012.
4. Heymann F, Trautwein C, Tacke F. Monocytes and macrophages as cellular targets in liver fibrosis. *Inflamm Allergy Drug Targets*. 2009;8(4):307-318.
5. Wynn TA, Barron L. Macrophages: Master regulators of inflammation and fibrosis. *Semin Liver Dis*. 2010;30(3):245-257.
6. Duffield JS, Forbes SJ, Constandinou CM, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest*. 2005;115(1):56-65.
7. Fallowfield JA, Mizuno M, Kendall TJ, et al. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J Immunol*. 2007;178(8):5288-5295.
8. Gordon S, Martinez FO. Alternative activation of macrophages: Mechanism and functions. *Immunity*. 2010;32(5):593-604.
9. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004;25(12):677-686.
10. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci*. 2008;13:453-461.
11. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958-969.
12. Huang WC, Sala-Newby GB, Susana A, Johnson JL, Newby AC. Classical macrophage activation upregulates several matrix metalloproteinases through mitogen activated protein kinases and nuclear factor-kappaB. *PLoS One*. 2012;7(8):e42507.
13. Song E, Ouyang N, Horbelt M, Antus B, Wang M, Exton MS. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol*. 2000;204(1):19-28.
14. Huang WC, Sala-Newby GB, Susana A, Johnson JL, Newby AC. Classical macrophage activation upregulates several matrix metalloproteinases through mitogen activated protein kinases and nuclear factor-kappaB. *PLoS One*. 2012;7(8):e42507.
15. Lopez-Navarrete G, Ramos-Martinez E, Suarez-Alvarez K, et al. Th2-associated alternative kupffer cell activation promotes liver fibrosis without inducing local inflammation. *Int J Biol Sci*. 2011;7(9):1273-1286.
16. Gibbons M, MacKinnon A, Ramachandran P, et al. Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis. *Am J Respir Crit Care Med*. 2011;184(5):569-581.
17. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: Therapeutic translation for fibrotic disease. *Nat Med*. 2012;18(7):1028-1040.
18. Boorsma CE, Draijer C, Melgert BN. Macrophage phenotypes in lung diseases. . in press.
19. Lopez-Guisa JM, Cai X, Collins SJ, et al. Mannose receptor 2 attenuates renal fibrosis. *J Am Soc Nephrol*. 2012;23(2):236-251.
20. Pesce JT, Ramalingam TR, Mentink-Kane MM, et al. Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. *PLoS Pathog*. 2009;5(4):e1000371.
21. Krausgruber T, Blazek K, Smallie T, et al. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol*. 2011;12(3):231-238.
22. Raes G, Van den Bergh R, De Baetselier P, et al. Arginase-1 and Ym1 are markers for murine, but not human, alternatively activated myeloid cells. *J Immunol*. 2005;174(11):6561; author reply 6561-2.
23. Raes G, De Baetselier P, Noel W, Beschin A, Brombacher F, Hassanzadeh Gh G. Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J Leukoc Biol*. 2002;71(4):597-602.
24. Van Gorp H, Delputte PL, Nauwynck HJ. Scavenger receptor CD163, a jack-of-all-trades and potential

target for cell-directed therapy. *Mol Immunol*. 2010;47(7-8):1650-1660.

25. Melgert BN, ten Hacken NH, Rutgers B, Timens W, Postma DS, Hylkema MN. More alternative activation of macrophages in lungs of asthmatic patients. *J Allergy Clin Immunol*. 2011;127(3):831-833.

26. Martinez FO, Helming L, Mueller R, et al. Identification and conserved resting and alternative activation signatures in human and mouse macrophages. . in press.

27. Louvet A, Teixeira-Clerc F, Chobert MN, et al. Cannabinoid CB2 receptors protect against alcoholic liver disease by regulating kupffer cell polarization in mice. *Hepatology*. 2011;54(4):1217-1226.

28. Chu D, Du M, Hu X, Wu Q, Shen J. Paeoniflorin attenuates schistosomiasis japonica-associated liver fibrosis through inhibiting alternative activation of macrophages. *Parasitology*. 2011;138(10):1259-1271.

29. Beljaars L, Molema G, Weert B, et al. Albumin modified with mannose 6-phosphate: A potential carrier for selective delivery of antifibrotic drugs to rat and human hepatic stellate cells. *Hepatology*. 1999;29(5):1486-1493.

30. Iredale JP. Models of liver fibrosis: Exploring the dynamic nature of inflammation and repair in a solid organ. *J Clin Invest*. 2007;117(3):539-548.

31. Hanania R, Sun HS, Xu K, Pustylnik S, Jeganathan S, Harrison RE. Classically activated macrophages use stable microtubules for matrix metalloproteinase-9 (MMP-9) secretion. *J Biol Chem*. 2012;287(11):8468-8483.

32. Wan J, Benkdane M, Teixeira-Clerc F, et al. Alternatively activated M2 kupffer cells promote selective killing of M1-polarized kupffer cells: A novel protective mechanism against alcoholic liver disease. *Hepatology*. 2011;54:1162A-1163A.

33. Belkin AM. Extracellular TG2: Emerging functions and regulation. *FEBS J*. 2011;278(24):4704-4716.

34. Fesus L, Piacentini M. Transglutaminase 2: An enigmatic enzyme with diverse functions. *Trends Biochem Sci*. 2002;27(10):534-539.

35. Fesus L, Szondy Z. Transglutaminase 2 in the balance of cell death and survival. *FEBS Lett*. 2005;579(15):3297-3302.

36. Popov Y, Sverdlov DY, Sharma AK, et al. Tissue transglutaminase does not affect fibrotic matrix stability or regression of liver fibrosis in mice. *Gastroenterology*. 2011;140(5):1642-1652.

37. Madala SK, Pesce JT, Ramalingam TR, et al. Matrix metalloproteinase 12-deficiency augments extracellular matrix degrading metalloproteinases and attenuates IL-13-dependent fibrosis. *J Immunol*. 2010;184(7):3955-3963.

38. Dalli J, Serhan CN. Specific lipid mediator signatures of human phagocytes: Microparticles stimulate macrophage efferocytosis and pro-resolving mediators. *Blood*. 2012;120(15):e60-72.

39. Morimoto K, Janssen WJ, Terada M. Defective efferocytosis by alveolar macrophages in IPF patients. *Respir Med*. 2012;106(12):1800-1803.

CHAPTER 3

Prostaglandin E_2 and Interferon γ affect liver fibrosis by altering the M1 and M2 macrophage balance in CCl_4-induced fibrogenesis

Marlies Schippers, Ruchi Bansal, Eduard Post, Catharina Reker-Smit, Barbro Melgert, Leonie Beljaars, Klaas Poelstra

Department of Pharmacokinetics, Toxicology and Targeting, University of Groningen, The Netherlands

ABSTRACT

During liver injury, various inflammatory cytokines activate resident macrophages and hepatic stellate cells (HSC). While a short, inflammatory response is beneficial to restore the liver architecture, uncontrolled continuation of this process may lead to fibrosis. Fibrosis is characterized by scarring of the liver and HSC are the main producers of this scar-tissue. Nowadays, there is growing evidence that macrophages also play an important role in the regulation of scar tissue formation. Macrophages can polarize into classically activated macrophages (M1) and alternative activated macrophages (M2), both with opposite activities on scar-tissue formation. In the present study we examined the hepatic M1/M2 composition during fibrogenesis and studied changes in macrophage polarization in mice treated with endogenous mediators of fibrosis and inflammation i.e. prostaglandin E_2 (PGE_2) and Interferon γ (IFNγ). We found a very localized accumulation of CD68+ cells in the acute and chronic phase of fibrogenesis, both comprising IRF5+ (M1) and YM1+ (M2) macrophages. This M1/M2 balance was changed during disease progression and PGE_2 as well as IFNγ affected the macrophage profile significantly; in the acute phase both mediators stimulated the local accumulation of M2 macrophages, associated with pro-fibrotic activities, whereas in the chronic phase they induced anti-fibrogenic activities associated with a down regulation of M2 macrophages.

Conclusion: Our results show a significant correlation between intrahepatic M2 accumulation and collagen deposition *in vivo*. PGE_2 and IFNγ both affect the macrophage polarisation *in vivo*, thereby stimulating repair mechanisms in the early phase and fibrolysis in the late phase of disease. It is hypothesized that in both phases these endogenous mediators steer the process towards homeostasis.

INTRODUCTION

Liver fibrosis is the result of a chronic inflammatory process in response to damage and the arrangement of cell types in the liver greatly determines hepatic responses to liver injury[1,2]. In the early, acute phase of fibrogenesis, inflammation and the wound healing process are beneficial to restore tissue damage but uncontrolled continuation of these processes may lead to fibrosis[3,4]. Fibrosis is characterized by scarring of the liver and hepatic stellate cells (HSC) are the main producers of scar tissue. The resident macrophages of the liver as well as newly recruited macrophages are known to play an important role in the activation of HSC[4-8]. The group of Iredale showed opposite roles of macrophages in the acute and repair phase of fibrogenesis which were related to MMP13-producing scar-associated macrophages[9,10]. These opposite roles of macrophages in inflammation and fibrosis might be explained by macrophage polarization.

Macrophages can polarize into at least two different phenotypes; classical activated macrophages (M1) and alternative activated macrophages (M2)[8,11-13]. M1 macrophages have microbicidal or tumoricidal capacities, are activated by IFNγ and TNFα or LPS, secrete pro-inflammatory cytokines[7,11,14] and release metalloproteinases (MMPs) that degrade scar tissue[15,16]. In contrast to M1, M2 macrophages have immunosuppressive actions, induce wound healing by fibroblast activation and secrete tissue remodelling enzymes including tissue inhibitors of metalloproteinase (TIMPs) and precursors for ECM proteins[4,7,11,16,17]. These M1 and M2 populations may be further divided into more subtypes[12,13], but to date, specific markers for these subpopulations are not known. For instance, one subpopulation of M2 macrophages are the M2-like macrophages, also called M2c or regulatory macrophages. Regulatory macrophages are generated in response to prostaglandins, glucocorticoids, immune-complexes and IL10, have anti-inflammatory activities by the secretion of IL10, TGFβ and PGE_2 and promote Treg responses[12,13]. It is unclear whether Treg cells have pro-or anti-fibrotic actions, since both activities are described in literature[18-22].

In atherosclerosis[23], alcoholic liver disease[24] renal[25] and lung fibrosis[26] as well as in other chronic inflammatory processes like, encephalomyelitis[27] or carcinogenetic processes[28], M1 and M2 macrophages appear to play an important role in disease progression. Several recent studies demonstrate a close association between M1/M2 macrophage accumulation and fibrogenesis[29,30], or resolution of fibrosis[9,10,31,32] but factors that regulate macrophage polarisation *in vivo* are unknown. In the present study we therefore examined the M1 and M2 composition after treatment with two endogenous mediators of inflammation and fibrosis i.e. IFNγ and PGE_2 at a very early stage after injury and after chronic injury. Both mediators are known to have antifibrotic activities, but their effect on the M1/M2 balance is unknown. In addition, IFNγ is endowed with strong pro-inflammatory activities, with the macrophage as a major target cell[13,33]. PGE_2 profoundly influences the inflammatory process too, exerting both pro-inflammatory and anti-inflammatory actions on macrophages[34-36]. Both endogenous mediators are produced in damaged areas[37,38].

Our study shows that the M1/M2 profile in the acute, inflammatory phase differs from the macrophage profile in the chronic phase of liver fibrosis. This macrophage balance was shifted, directly or indirectly, by IFNγ and PGE_2. Both mediators stimulate repair mechanisms in the early phase yet induce fibrolysis in the late phase of disease, apparently to steer the process towards homeostasis.

MATERIAL & METHODS

Materials

The following primary antibodies were used: goat anti-MMP-13, goat anti-TIMP-1 and goat anti-Desmin (Santa Cruz Biotecnology, Heidelberg, Germany), goat anti-chitinase 3-like/ECF-L (YM-1), rat anti-CD68 (AbD Serotec, Dusseldorf, Germany), rabbit anti-IRF5 (Protein Tech, Manchester, UK), mouse anti-PAR (BD Biosciences, Breda, Netherlands), Species-specific horseradish peroxidase-conjugated secondary antibodies were purchased from Dako Denmark A/S (Glostrup, Denmark).

Animal Experiments

All animal experiments were approved by the Animal Ethics Committee of the University of Groningen, the Netherlands. Animals were purchased from Harlan (Zeist, Netherlands).

CCl_4-induced acute liver fibrosis model: Male balb/c mice (20-22 g) received a single injection of CCl_4 diluted in olive oil (0.5 ml/kg). After 3h and 13h mice were treated with PGE_2 (Cayman Chemicals, Ann Arbor, MI) (0.5 mg/kg), IFNγ (5µg/mouse) or saline vehicle (n=6-7 per group). Mice were sacrificed 24h after CCl_4 injection.

CCl_4-induced advanced liver fibrosis model: Male balb/c mice (20-22 g) were injected twice a week intraperitoneally with saline or increasing doses of CCl_4 diluted in olive oil (week 1, 0.5 mL/kg; week 2, 0.8 mL/kg and week 3-8, 1 mL/kg prepared in olive oil). At week 7 and 8, mice were treated intravenously with PGE_2 (0.5 mg/kg, thrice per week) or saline vehicle, n = 6-9 per group. All mice were sacrificed at week 8.

Immunohistochemistry and quantitative analysis of sections: Immunohistochemistry was performed on 4 µm cryostat sections according to standard indirect immunoperoxidase methods. Stainings were visualized using 3-amino-9-ethylcarbazole or NovaRed (Vector Laboratories). Nuclei were counterstained with Mayer's hematoxylin. Immunohistochemical stainings were quantified by analysing complete sections from 3 different liver lobes of each animal at magnification 10x10 using the Cell D image analysing software (Olympus, Hamburg, Germany).

Western Blot Analysis: Liver homogenates were subjected to SDS-PAGE (12%) and the separated proteins were transferred to PVDF membrane. The membranes were blocked with TBST (20 mM TrisHCl, pH7.6, 154 mM NaCl, 0.1% Tween20) containing 5% skimmed milk and incubated with primary antibody. After washings, the blots were incubated with HRP-conjugated secondary anti-bodies (DAKO, Glostrup, Denmark). Finally, the blots were developed using Western Lightning-ECL reagent (Perkin-Elmer, Boston, MA) according to the manufacturer's instructions. Blots were quantified with Genetools (Syngene, Cambridge, UK). Samples from 3 different liver lobes of each animal were analyzed.

Quantitative Real Time PCR: Total RNA from liver tissue was isolated by RNeasy mini kit (Qiagen, Hilden, Germany) and reverse transcribed using cDNA synthesis kit (Promega). The primers of IL6 (forward: TGATGCTGGTGACAACCACGGC, reverse: TAAGCCTCCGACTTGTGAAGTGGTA), TNFα (forward: CATCTTCT-CAAAATTCGAGTGACAA, reverse: GAGTAGACAAGGTACAACCC), IL1β (forward:GCCAAGACAGGTCGCTCAGGG, reverse: CCCCCACACGTTGACAGCTAGG), TGFβ (forward: AGGGCTACCATGCCAACTTC, reverse: GTTGGA-

CAACTGCTCCACCT), IL10 (forward: ATAACTGCACCCACTTCCCAGTC, reverse: CCCAAGTAACCCT-TAAAGTCCTGC) and β-actin (forward: ATCGTGCGTGACATCAAAGA, reverse: ATGCCACAGGATTCCATACC) for real-time quantitative PCR were purchased from Sigma Genosys (Haverhill, UK). Quantitative real time PCR was performed using SensiMix SYBR kit (Bioline, UK) and reactions were analyzed by ABI7900HT sequence detection system (Applied Biosystems, Foster City, California). The threshold cycles (Ct) were calculated and relative gene expression was analyzed after normalizing for β-actin, house-keeping gene.

Statistical analysis:

Results are expressed as the mean ± SD, unless otherwise specified. Statistical analyses were performed using the Mann-Whitney t test. $p < 0.05$ was considered as the minimum level of significance.

RESULTS

M1/M2 expression in the acute and chronic phase of the CCl_4-induced liver damage model

We examined HSC (Desmin staining) and macrophages (CD68 staining) at the very early phase after injury and after long-term injury. A single dose of CCl_4 (acute injury) led to centrizonal necrosis after 24 hr, while 16 doses of CCl_4 during 8 weeks (chronic injury) led to activation and proliferation of HSC and extensive septa formation (fig.1a). Macrophages rapidly accumulated on the edges of the damaged areas and in the fibrotic bands during respectively the acute and chronic phase of disease (fig.1b). We used interferon-regulatory factor-5 (IRF-5) to identify the M1 macrophages[14]. IRF-5 is involved in the activation of genes encoding inflammatory cytokines such as IFNγ, TNFα, IL-6, IL12 and IL23[14]. Chitinase-like secretory protein (YM-1) was used to detect M2 macrophages. YM-1 is significantly upregulated after macrophage activation by IL4 and/or IL13[13,39,40]. IRF-5 and YM-1 both identify a major subset of CD68+ macrophages (fig.1c,d) and no co-localization of both markers was found. Expression of IRF-5 and YM-1 was clearly found around necrotic areas in mouse livers with acute liver damage and in fibrotic bands during established fibrosis. Western blot analysis confirmed that levels of M1 and M2 macrophage markers were significantly higher, 24 hr after CCl_4 administration compared to normal mice. However, in the chronic phase, levels of the M1 marker IRF5 were lower, whereas levels of M2 marker YM-1 were still significantly higher compared to control animals (fig.1c). So, during acute injury a balanced accumulation of both M1 and M2 macrophages was found, whereas during chronic injury the macrophage composition was shifted towards the profibrotic M2 macrophages.

During acute injury PGE_2 steers the M1/M2 balance towards the pro-fibrotic M2 phenotype.

Desmin and Protease-Activated Receptor-1 (PAR-1) are early fibrogenic markers that precede collagen deposition [41-43]. During acute liver injury, the number of desmin positive cells was not significantly changed by PGE_2 treatment (fig.2a). However, significantly higher levels for intrahepatic PAR-1 proteins were found after PGE_2 treatment as compared to untreated mice. In addition, a significant lower MMP13/TIMP-1 ratio was observed after PGE_2 treatment, which is known to favour ECM deposition (fig.2b). Subsequently, we studied whether these changes in fibrotic parameters were accompanied by changes in M1/M2 composition. In livers of CCl_4 mice, YM-1+ cells were located at the edges of necrotic fields. Higher numbers of YM-1+ cells were observed in PGE_2 treated mice compared to control CCl_4

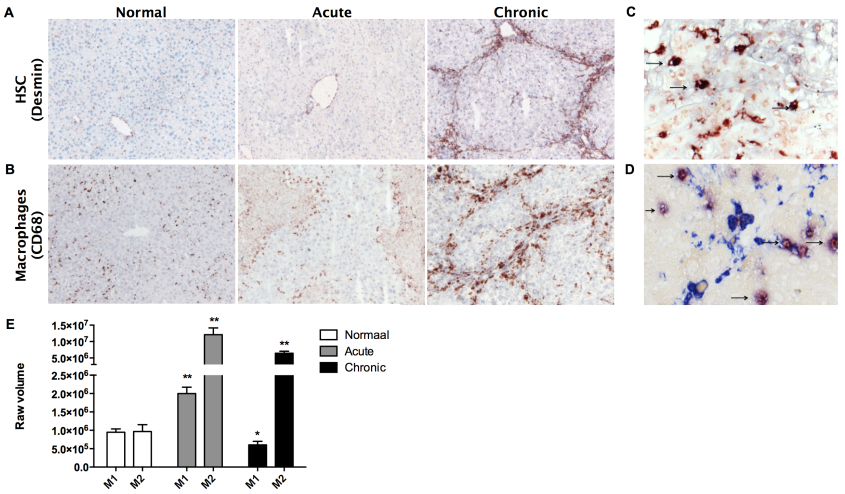

Figure 1. Localization of HSC and macrophages in the acute and chronic phase of CCl_4-induced liver fibrosis. Representative pictures of the staining for (**A**) HSC (desmin) and (**B**) macrophages (CD68) in livers of mice 24 hr and 8 weeks after the onset of CCl_4 administrations. Magnification, 40x. Representative pictures of double staining for (**C**) CD68 (red) and M1 marker IRF-5 (blue), and (**D**) CD68 (blue) and M2 marker YM-1(12, 33) (red) in fibrotic mouse livers. Arrows indicate double-positive cells (magnification 400x). (**E**) Western blot analysis of M1 and M2 macrophage accumulation in normal livers and livers of mice in the acute or chronic phase of fibrogenesis. Normal livers served as control. Bars represents mean ± SEM of 6-9 mice per group. *$P < 0.05$, **$P < 0.01$ versus untreated normal mice.

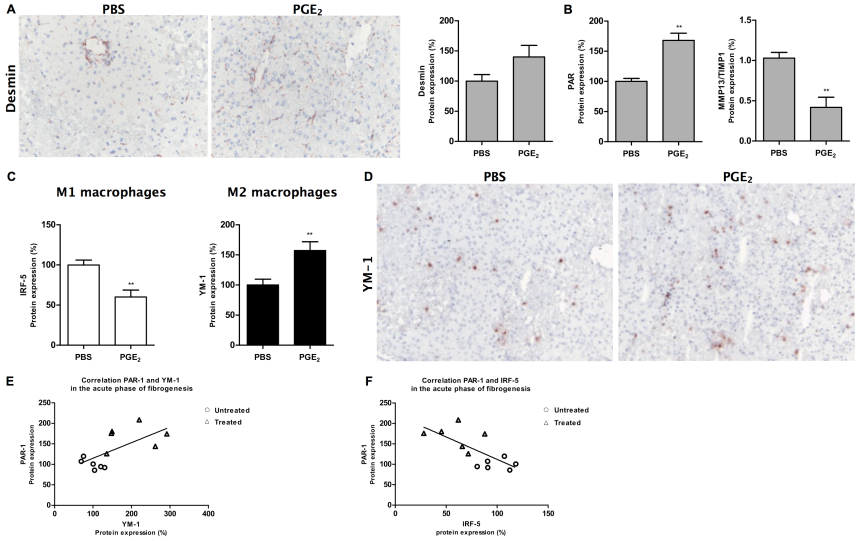

Figure 2. Effect of PGE_2 on the M1/M2 balance in the acute phase. (**A**) Representative pictures (magnification 100x) and quantification of desmin staining, 24 hr after CCl_4 administration in animals receiving either vehicle (control) or PGE_2, 3 and 13 hr after the CCl_4 injection. (**B**) Intrahepatic expression levels of the early fibrotic markers PAR-1 and MMP13/TIMP1-ratio after quantitative analysis of western blots. (**C**) Intrahepatic IRF-5 and YM-1 expression levels in different groups in the acute phase of fibrogenesis as analyzed by western blot. Figure **D** shows the localization of YM-1 positive cells by immunohistochemical staining, magnification 40x. Correlation between (**E**) PAR-1 and YM-1 expression (r=0.65, p<0.05) and (**F**) PAR-1 and IRF-5 (r=-0.71, p<0.01) in livers of different experimental groups. Bars represents mean ± SEM of 6 mice per group. **P < 0.01 versus CCl_4-treated mice.

mice. Also western blot analysis revealed significantly higher levels for M2 marker YM-1 in PGE_2-treated mice, whereas expression of M1 marker IRF5 was significantly lower in these mice relative to untreated CCl_4 mice (fig. 2c-d). A significant positive correlation between PAR-1 and M2 marker YM-1 was observed (r=0.65, p<0.05) (fig.2e), while a significant negative correlation between PAR-1 and M1 marker IRF-5 was found (r=-0.71, p<0.01) (fig.2f). To summarize, during acute liver injury, PGE_2 treatment shifted the macrophage pool towards the profibrotic M2 phenotype, which was associated with enhanced fibrogenesis.

During chronic injury PGE_2 shifts the M1/M2 balance towards the pro-inflammatory M1 phenotype

In the chronic phase of CCl_4 -induced liver fibrogenesis, PGE_2 treatment reduced expression levels for desmin compared to untreated fibrotic mice (fig.3a). This was associated with significant lower levels of PAR-1 and higher levels of MMP-13/TIMP-1 ratio, reflecting enhanced fibrinolysis (fig.3b). YM-1+ cells were localized along the fibrotic bands of the liver, and PGE_2 treatment resulted in significant lower expression levels of this M2 marker compared to untreated fibrotic animals whereas levels of M1 marker IRF5 were unchanged after PGE_2 treatment (fig.3c,d). A significant correlation between PAR-1 and YM-1+ cells was observed (r=0.59, p<0.05) (fig.3e). The relation between hepatic PAR-1 expression and fibrogenesis was illustrated by a significant correlation between PAR-1 protein expression levels and collagen deposition (r=0.49, p<0.05) (fig.3f). In summary, during chronic injury PGE_2 shifts the M1/M2 profile to the pro-inflammatory M1 phenotype, associated with a significant reduction in fibrotic parameters.

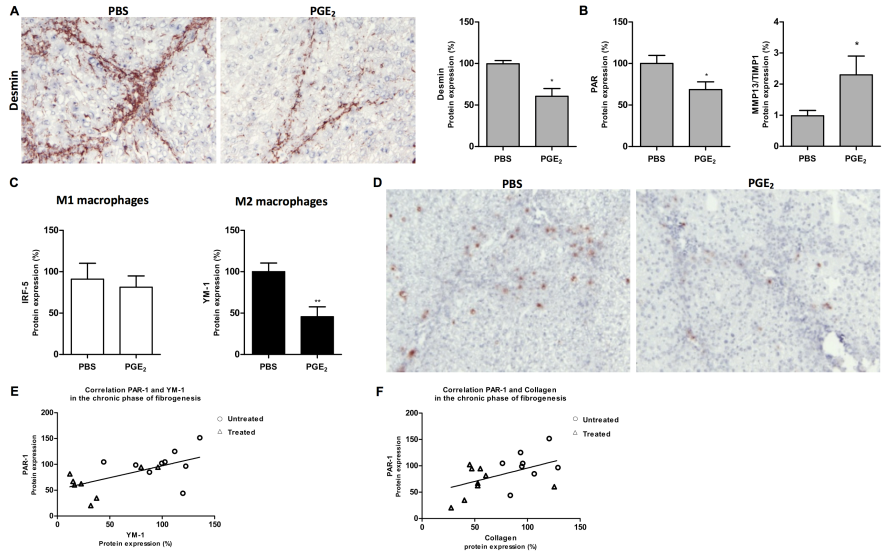

Figure 3; Effect of PGE_2 on the M1/M2 balance in the chronic phase. (**A**) Representative pictures (magnification 100x) and quantification of hepatic desmin staining after 8 weeks of treatment with CCl_4 of animals receiving either vehicle (control) or PGE_2 from week 6 to week 8. (**B**) Expression of the fibrotic markers PAR-1 and MMP13/TIMP1-ratio as analyzed by western blot. (**C**) Intrahepatic IRF-5 and YM-1 expression levels after PGE_2 treatment in the chronic phase of fibrogenesis as analyzed by western blot. Figure **D** shows the localization of YM-1 positive cells after immunohistochemical staining in the different experimental groups, magnification 40x. Correlation between (**E**) PAR-1 and YM-1 expression levels in livers (r=0.59, p<0.05) and (**F**) PAR-1 and collagen (r=0.49, p<0.05). Bars represents mean ± SEM of 6-9 mice per group. $*P < 0.05$, $**P < 0.01$ versus CCl_4-treated mice.

The effect of PGE_2 on intrahepatic inflammation

To examine whether PGE_2-induced alterations in the M1/M2 composition were associated with changes in inflammation, we measured its effect on C-reactive protein (CRP) which is a well known serum marker for intrahepatic inflammation[44]. Effects of PGE_2 were related to the effects of IFNγ, which is a pro-inflammatory cytokine, with the macrophage as its main target cell.

After CCl_4-induced acute injury, serum CRP levels were significantly higher as compared to healthy control animals. PGE_2 treatment was associated with significant lower serum CRP-levels compared to untreated CCl_4 mice. Treatment with IFNγ did not affect CRP levels as compared to untreated CCl_4 mice (fig.4a). After 8 weeks treatment with CCl_4, serum CRP levels were significantly lower as compared to healthy control animals. These levels were significantly higher in animals receiving PGE_2 treatment. IFNγ-treatment induced no significant changes in serum CRP levels of fibrotic animals (fig.4b).

Effects of IFNγ on the M1/M2 balance and fibrogenesis

We subsequently examined the effects of IFNγ on macrophage polarization and fibrotic parameters using the same set-up as the studies with PGE_2. Similar to PGE_2, IFNγ induced significantly higher YM1 expression levels during acute injury compared to untreated fibrotic animals, as observed by immunohistochemical stainings and quantifications of western blots. No changes in IRF5 positive cells were observed (fig.5a). Again, the number of desmin positive cells was not significantly enhanced in this acute phase, compared to normal mice (fig.5b,c). In contrast to the acute phase, IFNγ treatment in the chronic phase was associated with significant lower YM1 expression levels, whereas IRF5 levels were again unchanged (fig.5d). This was paralleled by significantly lower levels of desmin positive cells (fig.5e,f) as analysed by immunohistochemical staining. So, IFNγ induces similar effects on M1/M2 polarisation and fibrogenesis as PGE_2. Both mediators affect these processes in the acute and chronic phase of disease in opposite directions.

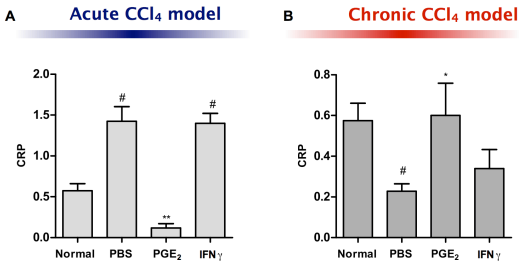

Figure 4. C-Reactive Protein (CRP) serum levels after PGE_2 or IFNγ treatment in the acute and chronic phase of CCl_4 induced fibrogenesis. Serum CRP-levels were measured (**A**) 24 hr after CCl_4 administration and (**B**) 8 weeks after the onset of CCl_4 injections in animals receiving either vehicle, PGE_2 or IFNγ treatment. Bars represents mean ± SEM of 6-9 mice per group. $^{\#}P < 0.05$ versus normal control mice. $^{**}P < 0.01$ versus CCl_4-treated mice.

PGE_2- and IFNγ-induced changes in M1/M2-associated cytokine mRNA profiles

Subsequently, we investigated cytokine production profiles in diseased livers after PGE_2 and IFNγ treatment. We focused on cytokines that are produced by M1 or M2 macrophages. No significant differences in mRNA levels of the examined cytokines were observed after acute liver injury and these levels were also not significantly altered by PGE_2 or IFNγ treatment (data not shown). In livers with established fibrosis, however, significantly higher mRNA levels for the M1-associated cytokines IL1ß, IL6 and TNFα were found after PGE_2 or IFNγ treatment as compared to livers of untreated fibrotic animals (fig.6a). In addi-

tion, levels of the Mreg-associated cytokines IL10 and TGFß[12,13] were significantly higher in PGE_2 and IFNγ-treated fibrotic mice (fig.6b) compared to untreated fibrotic animals.

The M1/M2 balance during fibrogenesis

To illustrate the relation between fibrogenesis and the M1/M2 composition we correlated collagen protein levels with protein levels of M1 marker IRF-5 and M2 marker YM-1 in all experimental groups. Results show no correlation between M1 accumulation and collagen expression, but a strong correlation between M2 accumulation and collagen deposition was found in the fibrotic livers (r=0.43, p<0.05, fig 6c).

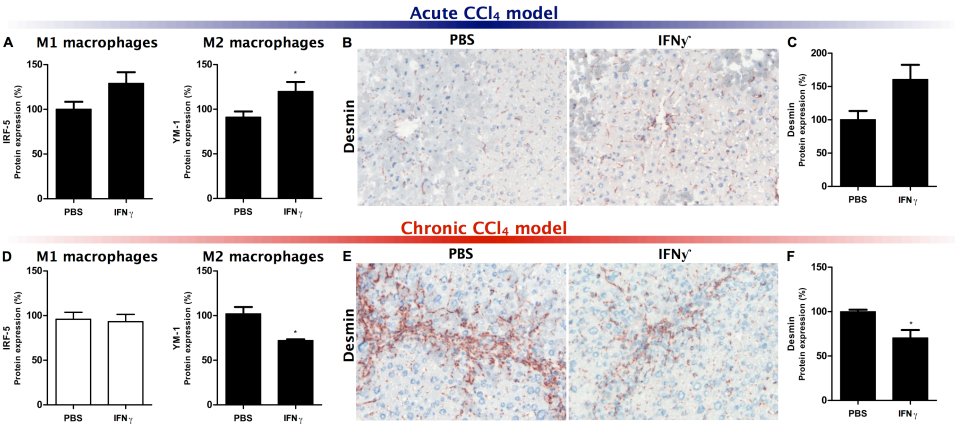

Figure 5. Effect of IFNγ on fibrogenesis in the acute phase after liver damage (24 hr studies) and in the chronic phase of fibrosis (8 weeks studies). (**A**) Western blot analysis of M1 and M2 macrophages in livers 24 hr after injection of CCl_4 or (**D**) 8 weeks after the onset of CCl_4 injections of mice receiving treatment with vehicle (control) or IFNγ. Representative pictures and quantification of desmin in the (**B,C**) acute and (**E,F**) chronic phase of fibrogenesis, magnification 100x. Bars represents mean ± SEM of 6 mice per group. *P < 0.05 versus CCl_4-treated mice.

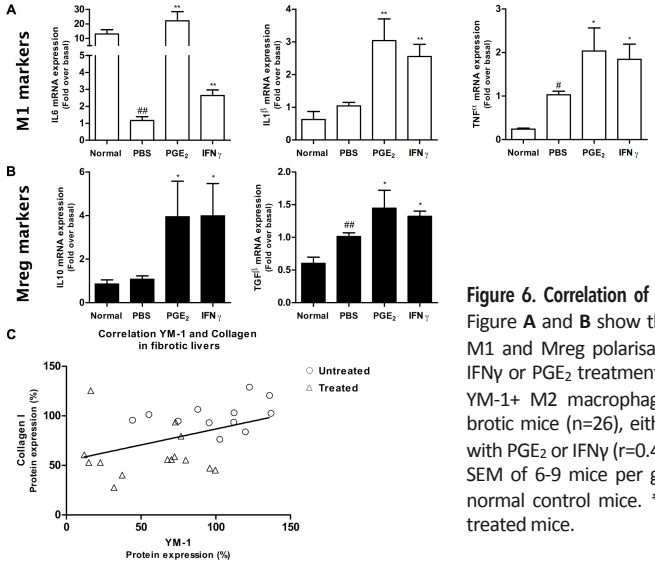

Figure 6. Correlation of YM-1 and collagen in fibrotic livers. Figure **A** and **B** show the **c**ytokine profiles that affect the M1 and Mreg polarisation in fibrotic livers after vehicle, IFNγ or PGE_2 treatment. (**C**) Correlation between influx of YM-1+ M2 macrophages and collagen deposition in fibrotic mice (n=26), either treated with vehicle or treated with PGE_2 or IFNγ (r=0.43, p<0.05). Bars represents mean ± SEM of 6-9 mice per group. #P < 0.05, ##P < 0.01 versus normal control mice. *P < 0.05, **P < 0.01 versus CCl_4-treated mice.

DISCUSSION

This study demonstrates the presence of classically activated macrophages (M1) and alternatively activated macrophages (M2) in the acute, inflammatory phase and the chronic phase of liver fibrogenesis *in vivo*. For the first time, we showed that the intrahepatic M1/M2 profile changes in opposite directions in the acute and chronic phase of liver fibrogenesis by the endogenous inflammatory mediators PGE_2 and IFNγ. During the acute phase of fibrogenesis, M1 macrophages secrete, together with hepatocytes and HSC, matrix metalloproteinases (MMP) to allow efficient tissue access for inflammatory cells at sites of injury and to promote tissue remodelling[4,7,11,45,46]. This process is counterbalanced by M2 macrophages, that stimulate HSC activation and wound healing[7,11,12,33]. During the acute phase of liver fibrosis we found significant higher levels of M1 marker IRF-5 and M2 marker YM-1 as compared to normal livers. However, in the late, chronic phase of fibrogenesis the pool of M1/M2 macrophages shifted towards the profibrotic M2 phenotype resulting in a dominant wound healing process and fibrosis. This indicates that the tissue microenvironment may regulate the polarization of macrophages which is also in line with recent evidence that the amino-acid metabolism is important in shaping the functional phenotype of macrophages in response to polarizing stimuli[30]

To understand the role of the M1 and M2 macrophages during acute and chronic fibrogenesis in more detail, we examined M1/M2 profiles in mouse livers after treatment with endogenous mediators PGE_2 or IFNγ and correlated these macrophage numbers with wound repair mechanisms. Although it is well known that the cyclo-oxygenase product PGE_2 affects inflammatory processes, its effect on the polarisation of macrophages in M1 and M2 phenotypes during liver fibrosis is unclear. *In vitro,* PGE_2 was found to induce an M2 phenotype in tumor-associated macrophages[47], and in human mesenchymal stem/stromal cells endogenous PGE_2 stimulated polarisation of macrophages into the M2-subtype[48]. In addi-

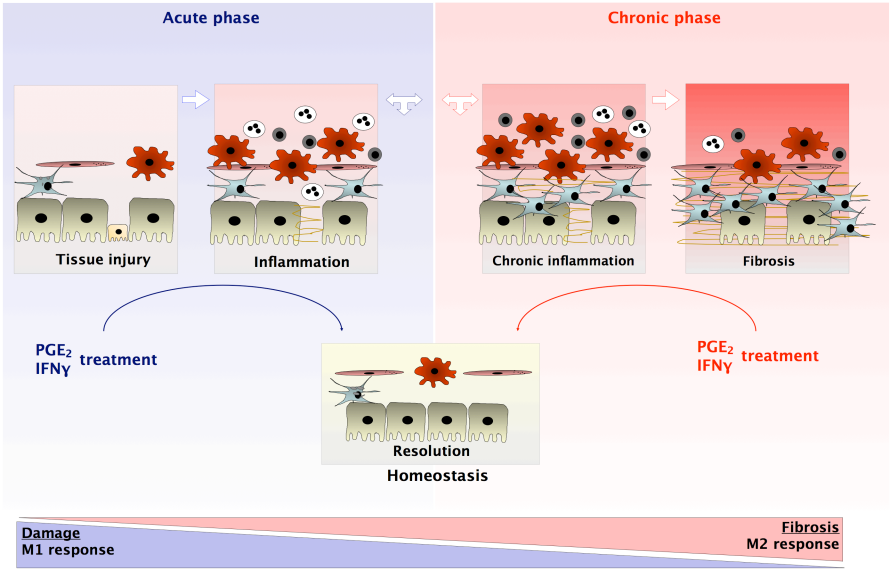

Figure 7. Schematic overview of the effects of PGE_2 and IFNγ in the acute and chronic phase after liver damage. PGE_2 and IFNγ induce in both phases a Th1 response within the liver. In the acute phase after liver damage this results in inflammation followed by wound healing whereas in the chronic phase this leads to degradation of the ECM. In both cases this results in resolution of the distorted liver architecture.

tion, PGE_2 is known to regulate immune responses[49], it suppresses cytokine production[50], and regulates anti-inflammatory actions in alveolar macrophages[51].

We found that treatment of mice with PGE_2 induced opposite effects during acute and chronic injury: while during acute injury the M1/M2 profiles shifted towards M2 by PGE_2, during chronic injury this composition shifted towards M1. In the acute phase of liver fibrosis, the observed macrophage shift towards the anti-inflammatory M2 subtype by PGE_2 was associated with enhanced wound healing. This protective effect of a polarized Th2-type response was also found during experimental helminth infection by Chen *et al*[52] and is essential to restore tissue damage. However, in the chronic phase PGE_2 shifted the M1/M2 profile towards M1, associated with changes towards attenuated fibrogenesis. Apparently, PGE_2 induces in the late phase a more inflammatory profile, associated with ECM degradation. This is supported by studies of Yao *et al.* who found that PGE_2 signalling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion[35]. Our data also support the dual effects of macrophage depletion in acute liver injury and fibrosis found by Duffield *et al*[9,10]. The scar-associated macrophages which were responsible for the resolution of liver fibrosis through the production of MMP13 found by Fallowfield *et al*[10], most likely represent macrophages from the M1 phenotype which are known to produce MMPs[4,16].

To examine whether PGE_2-induced alterations in the M1/M2 balance were associated with changes in intrahepatic inflammation, we measured serum CRP levels. The results confirmed the anti-inflammatory effect of PGE_2 in the acutely damaged livers, and the pro-inflammatory effect of this mediator in the chronically affected livers (fig.4). Parallel we tested IFNγ with known pro-inflammatory effects on macrophages[35,43]. IFNγ did not significantly alter CRP levels in the acute phase, nor in the chronic phase compared to fibrotic mice receiving vehicle. So, IFNγ at this dose did not affect intrahepatic inflammation. This prompted us to examine whether IFNγ induced similar alterations in the M1/M2 balance and fibrogenesis as PGE_2. Despite their different pleiotropic effects on the immune system[13,33-36], IFNγ and PGE_2 displayed identical effects on the M1/M2 balance and fibrogenesis. PGE_2 and IFNγ both affected macrophage polarisation *in vivo*, thereby stimulating repair mechanisms in the early phase and fibrolysis in the late phase of disease. As illustrated in figure 7, these opposite effects may be explained by assuming that PGE_2 and IFNγ drive the different repair mechanisms towards homeostasis. Immediately after damage, restricted inflammation and effective wound healing are essential to preserve liver function. However, when the pathogenic process has led to a situation of excessive ECM deposition with dearrangement of the liver architecture, PGE_2 and IFNγ enhance inflammation, reduce fibrogenesis and stimulate ECM degradation, again leading to resolution of damage and thus to homeostasis.

The relation between the composition of M1 and M2 macrophages and fibrogenesis is also illustrated by the strong correlation between the intrahepatic accumulation of YM-1+ cells and the intrahepatic collagen deposition (fig.6a). Also in chronic kidney injury models a polarisation of inoculated macrophages from M1 to M2 was seen in the repair phase[25]. Whether the Mreg-phenotype is also involved in this process remains to be established. The (yet unidentifiable) Mreg produces pro-fibrotic, but anti-inflammatory cytokines like IL10 and TGFβ[12,13], which were both up regulated by PGE_2 and IFNγ (fig.6). It has been shown that macrophages that accumulate in tissue during resolution of fibrosis produce anti-inflammatory cytokines including IL10[53] and have hybrid M1/M2 functions controlled by cAMP[54]. Of note, this Mreg phenotype also produces PGE_2[8], which can induce the effects shown in this paper. It is anticipated that future developments will include the discovery of new markers, and new phenotypes

may be found. For instance, Ramachandran *et al*. recently identified the restorative macrophages as a new phenotype apart from the M1 and M2 macrophages, recruited from inflammatory monocytes[5]. Our study provides insight in the role and the regulation of the two major subsets known today.

Collectively, our study shows the effects of M1/M2 polarisation during fibrogenesis, and identifies the M1/M2 balance as a relevant target for therapies. Most importantly, profile of cells that are actually present in the diseased affect the outcome of therapies different in the early or late stage. Collectively, these data show that complex cell-cell interactions[1,2,4,7,55] in different phases of disease are key factors that determine disease activity and therapy outcome.

REFERENCES

1. Puche JE, Lee YA, Jiao J, et al. A novel murine model to deplete hepatic stellate cells uncovers their role in amplifying liver damage. *Hepatology*. 2012.
2. Barron L, Wynn TA. Fibrosis is regulated by Th2 and Th17 responses and by dynamic interactions between fibroblasts and macrophages. *Am J Physiol Gastrointest Liver Physiol*. 2011;300(5):G723-8.
3. Pinzani M, Rombouts K, Colagrande S. Fibrosis in chronic liver diseases: Diagnosis and management. *J Hepatol*. 2005;42 Suppl(1):S22-36.
4. Heymann F, Trautwein C, Tacke F. Monocytes and macrophages as cellular targets in liver fibrosis. *Inflamm Allergy Drug Targets*. 2009;8(4):307-318.
5. Ramachandran P, Pellicoro A, Vernon MA, et al. Differential ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci U S A*. 2012.
6. Thomas JA, Pope C, Wojtacha D, et al. Macrophage therapy for murine liver fibrosis recruits host effector cells improving fibrosis, regeneration, and function. *Hepatology*. 2011;53(6):2003-2015.
7. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol*. 2004;4(8):583-594.
8. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003;3(1):23-35.
9. Duffield JS, Forbes SJ, Constandinou CM, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest*. 2005;115(1):56-65.
10. Fallowfield JA, Mizuno M, Kendall TJ, et al. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J Immunol*. 2007;178(8):5288-5295.
11. Laskin DL, Sunil VR, Gardner CR, Laskin JD. Macrophages and tissue injury: Agents of defense or destruction? *Annu Rev Pharmacol Toxicol*. 2011;51:267-288.
12. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004;25(12):677-686.
13. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958-969.
14. Krausgruber T, Blazek K, Smallie T, et al. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol*. 2011;12(3):231-238.
15. Hemmann S, Graf J, Roderfeld M, Roeb E. Expression of MMPs and TIMPs in liver fibrosis - a systematic review with special emphasis on anti-fibrotic strategies. *J Hepatol*. 2007;46(5):955-975.
16. Song E, Ouyang N, Horbelt M, Antus B, Wang M, Exton MS. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol*. 2000;204(1):19-28.
17. Cook PC, Jones LH, Jenkins SJ, Wynn TA, Allen JE, Macdonald AS. Alternatively activated dendritic cells regulate CD4+ T-cell polarization in vitro and in vivo. *Proc Natl Acad Sci U S A*. 2012;109(25):9977-9982.
18. Claassen MA, de Knegt RJ, Tilanus HW, Janssen HL, Boonstra A. Abundant numbers of regulatory

T cells localize to the liver of chronic hepatitis C infected patients and limit the extent of fibrosis. *J Hepatol*. 2010;52(3):315-321.

19. Kanellakis P, Dinh TN, Agrotis A, Bobik A. CD4(+)CD25(+)Foxp3(+) regulatory T cells suppress cardiac fibrosis in the hypertensive heart. *J Hypertens*. 2011;29(9):1820-1828.

20. Kotsianidis I, Nakou E, Bouchliou I, et al. Global impairment of CD4+CD25+FOXP3+ regulatory T cells in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2009;179(12):1121-1130.

21. Liu F, Liu J, Weng D, et al. CD4+CD25+Foxp3+ regulatory T cells depletion may attenuate the development of silica-induced lung fibrosis in mice. *PLoS One*. 2010;5(11):e15404.

22. Kitani A, Fuss I, Nakamura K, Kumaki F, Usui T, Strober W. Transforming growth factor (TGF)-beta1-producing regulatory T cells induce smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF-beta1-mediated fibrosis. *J Exp Med*. 2003;198(8):1179-1188.

23. Gordon S, Martinez FO. Alternative activation of macrophages: Mechanism and functions. *Immunity*. 2010;32(5):593-604.

24. Louvet A, Teixeira-Clerc F, Chobert MN, et al. Cannabinoid CB2 receptors protect against alcoholic liver disease by regulating kupffer cell polarization in mice. *Hepatology*. 2011;54(4):1217-1226.

25. Lee S, Huen S, Nishio H, et al. Distinct macrophage phenotypes contribute to kidney injury and repair. *J Am Soc Nephrol*. 2011;22(2):317-326.

26. Lekkerkerker AN, Aarbiou J, van Es T, Janssen RA. Cellular players in lung fibrosis. *Curr Pharm Des*. 2012;18(27):4093-4102.

27. Denney L, Kok WL, Cole SL, Sanderson S, McMichael AJ, Ho LP. Activation of invariant NKT cells in early phase of experimental autoimmune encephalomyelitis results in differentiation of Ly6Chi inflammatory monocyte to M2 macrophages and improved outcome. *J Immunol*. 2012;189(2):551-557.

28. Heusinkveld M, de Vos van Steenwijk PJ, Goedemans R, et al. M2 macrophages induced by prostaglandin E2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells. *J Immunol*. 2011;187(3):1157-1165.

29. Ariel A, Timor O. Hanging in the balance: Endogenous anti-inflammatory mechanisms in tissue repair and fibrosis. *J Pathol*. 2013;229(2):250-263.

30. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol*. 2013;229(2):176-185.

31. Ramachandran P, Iredale JP. Macrophages: Central regulators of hepatic fibrogenesis and fibrosis resolution. *J Hepatol*. 2012;56(6):1417-1419.

32. Duffield JS, Lupher M, Thannickal VJ, Wynn TA. Host responses in tissue repair and fibrosis. *Annu Rev Pathol*. 2012.

33. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*. 2011;11(11):723-737.

34. Sheibanie AF, Khayrullina T, Safadi FF, Ganea D. Prostaglandin E2 exacerbates collagen-induced arthritis in mice through the inflammatory interleukin-23/interleukin-17 axis. *Arthritis Rheum*. 2007;56(8):2608-2619.

35. Yao C, Sakata D, Esaki Y, et al. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat Med*. 2009;15(6):633-640.

36. Sheibanie AF, Tadmori I, Jing H, Vassiliou E, Ganea D. Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. *FASEB J*. 2004;18(11):1318-1320.

37. Ruwart MJ, Wilkinson KF, Rush BD, et al. The integrated value of serum procollagen III peptide over time predicts hepatic hydroxyproline content and stainable collagen in a model of dietary cirrhosis in the rat. *Hepatology*. 1989;10(5):801-806.

38. Bansal R, Prakash J, Post E, Beljaars L, Schuppan D, Poelstra K. Novel engineered targeted interferon-gamma blocks hepatic fibrogenesis in mice. *Hepatology*. 2011;54(2):586-596.

39. Raes G, De Baetselier P, Noel W, Beschin A, Brombacher F, Hassanzadeh Gh G. Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J Leukoc Biol*. 2002;71(4):597-602.

40. Webb DC, McKenzie AN, Foster PS. Expression of the Ym2 lectin-binding protein is dependent on interleukin (IL)-4 and IL-13 signal transduction:

Identification of a novel allergy-associated protein. *J Biol Chem*. 2001;276(45):41969-41976.

41. Calvaruso V, Maimone S, Gatt A, et al. Coagulation and fibrosis in chronic liver disease. *Gut*. 2008;57(12):1722-1727.

42. Marra F, Grandaliano G, Valente AJ, Abboud HE. Thrombin stimulates proliferation of liver fat-storing cells and expression of monocyte chemotactic protein-1: Potential role in liver injury. *Hepatology*. 1995;22(3):780-787.

43. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: Therapeutic translation for fibrotic disease. *Nat Med*. 2012;18(7):1028-1040.

44. Peisajovich A, Marnell L, Mold C, Du Clos TW. C-reactive protein at the interface between innate immunity and inflammation. *Expert Rev Clin Immunol*. 2008;4(3):379-390.

45. Mann CJ, Perdiguero E, Kharraz Y, et al. Aberrant repair and fibrosis development in skeletal muscle. *Skelet Muscle*. 2011;1(1):21.

46. Cataldo DD, Tournoy KG, Vermaelen K, et al. Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation. *Am J Pathol*. 2002;161(2):491-498.

47. Liu L, Ge D, Ma L, et al. Interleukin-17 and prostaglandin E2 are involved in formation of an M2 macrophage-dominant microenvironment in lung cancer. *J Thorac Oncol*. 2012;7(7):1091-1100.

48. Ylostalo JH, Bartosh TJ, Coble K, Prockop DJ. Human mesenchymal Stem/Stromal cells cultured as spheroids are self-activated to produce prostaglandin E2 that directs stimulated macrophages into an anti-inflammatory phenotype. *Stem Cells*. 2012;30(10):2283-2296.

49. Kalinski P. Regulation of immune responses by prostaglandin E2. *J Immunol*. 2012;188(1):21-28.

50. Nataraj C, Thomas DW, Tilley SL, et al. Receptors for prostaglandin E(2) that regulate cellular immune responses in the mouse. *J Clin Invest*. 2001;108(8):1229-1235.

51. Ratcliffe MJ, Walding A, Shelton PA, Flaherty A, Dougall IG. Activation of E-prostanoid4 and E-prostanoid2 receptors inhibits TNF-alpha release from human alveolar macrophages. *Eur Respir J*. 2007;29(5):986-994.

52. Chen F, Liu Z, Wu W, et al. An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. *Nat Med*. 2012;18(2):260-266.

53. Biswas SK, Mantovani A. Orchestration of metabolism by macrophages. *Cell Metab*. 2012;15(4):432-437.

54. Bystrom J, Evans I, Newson J, et al. Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP. *Blood*. 2008;112(10):4117-4127.

55. Heymann F, Hammerich L, Storch D, et al. Hepatic macrophage migration and differentiation critical for liver fibrosis is mediated by the chemokine receptor C-C motif chemokine receptor 8 in mice. *Hepatology*. 2012;55(3):898-909.

CHAPTER 4

Prostaglandin E_2 affects fibrogenesis via modulation of intrahepatic Epac-1 expression in mice.

Marlies Schippers[1*], Martina Schmidt[2], Sophie Lotersztajn[3], Eduard Post[1], Karin Spijkers[1], Stijntje Boxum[1], Leonie Beljaars[1], Klaas Poelstra[1]

[1] Department of Pharmacokinetics, Toxicology and Targeting, University of Groningen, The Netherlands
[2] Groningen University Institute for Drug Exploration (GUIDE), Department of Molecular Pharmacology, University of Groningen, Groningen, The Netherlands
[3] INSERM, U 841, Créteil, F-94000 France

ABSTRACT

Recently, exchange protein activated by cAMP (Epac-1) was discovered as a new signaling mechanism for cAMP-mediated effects. Sofar, the contribution of Epac-1 in liver fibrogenesis *in vivo* is completely unknown. Activation of myofibroblasts, the collagen-producing cells during fibrogenesis, is tightly controlled by cAMP activity. Prostaglandin E_2 (PGE_2) is a lipid mediator produced by cyclooxygenase-2 (COX-2) and it inhibits myofibroblast activation *in vitro* by activation of cAMP. In this study we therefore used this endogenous mediator to explore the role of Epac-1 in a chronic CCl4-mouse model for liver fibrosis. Our data show that hepatic Epac-1 expression levels were significantly decreased in fibrotic livers of mice and man compared to normal livers. In fibrotic mouse livers, PGE_2 treatment normalized these Epac-1 levels which was paralleled by reduced collagen, vimentin, α-smooth muscle actin and pSMAD expression levels as well as Rho-kinase activity. The COX-2 inhibitor Niflumic acid was found to enhance Epac-1 expression and fibrogenesis. These studies show the antifibrotic effects of PGE_2 *in vivo* and identify Epac-1 as a major signaling pathway during liver fibrosis. Our studies also provide insight into the effects of COX inhibitors during liver fibrosis which represents an unknown field.

INTRODUCTION

Liver fibrosis is the result of an imbalanced tissue remodelling process due to chronic inflammation associated with excessive scar tissue formation and liver failure[1,2]. Hepatic stellate cells (HSC), key players in this process, transform from quiescent cells into proliferative, fibrogenic and contractile myofibroblast-like cells in response to growth factors such as transforming growth factor β (TGFβ) and platelet derived growth factor BB (PDGF-BB)[3,4]. The differentiation and activation of HSC is tightly controlled by second messenger cyclic AMP (cAMP) activity[5]. Increased intracellular cAMP levels inhibit fibroblast migration and proliferation and, moreover, block the phenotype switch into myofibroblasts leading to less scar tissue formation[6].

Prostaglandin E_2 (PGE_2) is a potent lipid mediator derived from the oxidation of arachidonic acid by cyclooxygenase-2 (COX-2) that regulates inflammatory processes and directly affects cAMP levels in most cell types. In fibroblasts, PGE_2 exerts anti-fibrotic activities through cAMP activation by binding to its EP_2 or EP_4 receptor[5,7-9]. Antifibrotic effects of a stable PGE_2 isoform were found in rats with liver fibrosis[10]. However, the mechanism behind this effect has not yet been extensively investigated probably due to its short half-life and rapid clearance[11]. Since COX inhibitors directly interfere with PGE_2 levels[12] and these drugs are widely used also in patients prone to fibrogenesis, the interrelationship between PGE_2 and fibrosis warrants further exploration.

Next to the classic cAMP effector Protein Kinase A (PKA), Exchange Protein Activated by cAMP (Epac) was recently discovered as a new signaling mechanism for cAMP-mediated effects[13]. Epac is a guanide nucleotide exchange factor (GEF), specific for the Ras family[14]. It is involved in the regulation of several cellular key processes such as calcium handling, neural signalling, inflammation, cell proliferation and migration, by promoting the exchange of GTP and GDP in the GTPase cycle[14,15]. Two isoforms of Epac are now identified; Epac-1, which is reported in the heart, vasculature, brain, kidney and lungs, and Epac-2, mostly found in brains and adrenal glands[15]. The key role of cAMP in cellular processes in fibroblasts suggests a prominent role for Epac-1 during fibrogenesis. However to date, nothing is known about the contribution of Epac-1 during fibrogenesis *in vivo* or even alterations in Epac-1 levels during liver fibrosis. A reduced Epac-1 expression by TGFβ was recently found *in vitro* in hepatic stellate cells[16], which again suggests a role for this mediator.

In this study, we therefore investigated the effect of PGE_2 on fibrogenesis and the mechanism of action of this cyclooxygenase product in a chronic CCl_4-induced mice model for liver fibrosis. This study provides insight into the direct antifibrotic effect of PGE_2 during liver fibrogenesis and we identify Epac-1 as a important intracellular signalling pathway for PGE_2-mediated anti-fibrotic effects *in vivo*.

MATERIAL & METHODS

Materials

The selective Epac-1 agonist (8-pCPT-2'-O-Me-cAMP) and selective PKA- agonist (6-Bnz-cAMP) were purchased from Biolog, Life Science Institute (Bremen, Germany), PGE_2 (Dinosprostone) was from Cayman Chemicals (Ann Arbor, MI), PDGF-BB was from PeproTech (Rocky Hill, NJ), Niflumic acid was from (MP Biomedicals, France), TGF-β was from Roche Diagnostics (Mannheim, Germany) and Transwell pla-

tes were from Corning Life Sciences (Lowell, MA). Primary antibodies were as follows: mouse anti-α-SMA and anti-Desmin (Sigma-Aldrich, St. Louis, MO), goat anti-collagen I (Southern Biotechnology Associates, Birmingham, AL), goat anti-pSMAD 2/3 and goat anti-Epac-1 (Santa Cruz Biotecnology, Heidelberg, Germany), goat anti-PKA mouse and anti-pMLC2 (Ser19) (Cell Signaling Technology, Danvers, MA). Species-specific horseradish peroxidase and FITC-/TRITC-conjugated secondary antibodies were purchased from Dako Denmark A/S (Glostrup, Denmark).

Cell experiments

Primary HSC were isolated from livers of male Wistar rats (>500 g, Harlan, Netherlands) according to previously published methods[17]. After isolation, HSC were cultured on plastic for 10 days until activation and then used for experiments.

Human hepatic stellate cells (LX2) were kindly provided by Prof. Scott Friedman (Mount Sinai Hospital, New York). LX2 were cultured in DMEM-Glutamax (Invitrogen) supplemented with 10% FBS. All *in vitro* data are presented as the mean of three different experiments performed in duplo.

Proliferation Assay: To examine the proliferation of primary HSC, cells were seeded in 12-well culture plates (2 x 10^5 cells per well) and grown for 2 days. Cells were starved overnight in serum-free medium and were incubated with 5 μM PGE_2, 50 μM Epac-1 agonist or 500 μM PKA agonist along with 50 ng/ml PDGF-BB. After 18h, 3H-thymidin (final concentration of 0.25 uCi/ml) was added for 6h. Subsequently, cells were washed with PBS, fixed with TCA 5% and lysed with 1M NaOH. Proliferation of HSC were counted with Optima Gold Scintillation fluid using a scintillation counter (Perkin Elmer).

rHSC Migration Assay: To examine the migration of primary HSC, 6×10^4 cells per well were added to the upper compartment of a Transwell chamber, together with 5 μM PGE_2, 50 μM Epac-1 agonist, or 500 μM PKA agonist. PDGF-BB (50 ng/ml) was added to the lower chamber to stimulate migration. The cells were then incubated for 24 h, fixed, and stained with Mayer's hematoxylin. Cells on both sides of the membrane were counted in five fields per membrane at 40× magnification. Migration was calculated as the percentage of cells on the lower side of the membrane relative to the total number of cells in each field.

LX2 Collagen-I deposition: To asses effects on fibrotic parameters in a human hepatic stellate cells, LX2 cells were starved for 24h and subsequently incubated with 5 μM PGE_2, 50 μM Epac-1 agonist, or 500 μM PKA agonist along with 5 ng/ml TGFβ for 24h. Cells were then fixed in aceton and stained for collagen using goat anti-Collagen I IgG and standard indirect immunoperoxidase methods.

Animal Experiments

All animal experiments were approved by the Animal Ethics Committee of the University of Groningen, the Netherlands. Animals were purchased from Harlan (Zeist, Netherlands).

CCl_4-Induced Liver Fibrosis Model: Male balb/c mice (20-22 g) were injected twice a week intraperitoneally with PBS or increasing doses of CCl_4 in olive oil (week 1, 0.5 ml/kg; week 2, 0.8 ml/kg and week 3-8, 1 ml/kg). At week 7 and 8, mice were treated intravenously with vehicle (PBS), PGE_2 (0.5 mg/kg) or Niflumic acid (5 mg/kg) thrice per week, n = 6-9 per group. Mice were sacrificed at week 8. Normal mice served as control (n=6).

Immunohistochemistry and quantitative analysis of sections: Immunohistochemistry was performed on 4-µm cryostat sections or cells according to standard methods. Immunofluorescent staining in sections was visualized using a M.O.M. kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Immunohistochemical stainings were quantitated by analysing complete sections from 3 different liver lobes of each animal at magnification 10x10 using the Cell D image analysing software (Olympus, Hamburg, Germany).

Western Blot Analysis: Liver homogenates were subjected to SDS-PAGE (12%) and proteins were transferred to PVDF membrane according to standard methods. Blots were developed using Western Lightning-ECL reagent (Perkin-Elmer, Boston, MA) according to the manufacturer's instructions and quantified with Genetools (Syngene, Cambridge, UK). Of each animal, samples from 3 different liver lobes were analyzed.

Quantitative Real Time PCR: RNA was isolated by RNeasy mini kit (Qiagen, Hilden, Germany) and reverse transcribed using cDNA synthesis kit (Promega). The primers of αSMA (forward: ACTACTGCCGAGCGTGAGAT, reverse: CCAATGAAAGATGGCTGGAA), Çollagen 1a1 (forward: TGACTGGAAGAGCGGAGAGT, reverse: ATCCATCGGTCATGCTCTCT) and β-actin (forward: ATCGTGCGTGACATCAAAGA, reverse: ATGCCACAGGATTCCATACC) for real-time quantitative PCR were purchased from Sigma Genosys (Haverhill, UK). Quantitative real time PCR was performed using SensiMix SYBR kit (Bioline, UK) and reactions were analyzed by ABI7900HT sequence detection system (Applied Biosystems, Foster City, California). The threshold cycles (Ct) were calculated and relative gene expression was analyzed after normalizing for β-actin, house-keeping gene.

Human tissue

Human cirrhotic liver tissue (n=6) was obtained from patients undergoing liver transplantation and was anonymously provided by University Medical Center Groningen, The Netherlands. Normal human liver tissue (n=6) was obtained from residual liver tissue after partial hepatectomy. All procedures were performed according to the Dutch Code of Conduct (http://www.federa.org/gedragscodes-codes-conduct-en).

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using the Mann-Whitney *t* test. $p < 0.05$ was considered as significant.

RESULTS

PGE_2 induces anti-fibrotic effects *in vitro.*

The effects of PGE_2 on fibrogenesis *in vitro* were examined by analysis of migration, proliferation, and collagen I protein expression in cultures of primary isolated rat hepatic stellate cells (rHSC) and in human LX2 cells (n=3 per experiment). A significant reduction of PDGF-stimulated migration and ^{3}H-thymidine incorporation was observed in rHSC after incubation with PGE_2 (fig.1a-b). PGE_2 attenuated PDGF-induced migration by 60% and abolished PDGF-induced proliferation in rHSC. Immunohistochemical analysis also

showed that PGE_2 administration to human LX2 cells significantly inhibited the TGFβ-induced collagen-1 protein production (fig.1c).

PGE_2 induces anti-fibrotic effects *in vivo*.

We first examined the effects on α-smooth muscle actin (α-SMA) and vimentin expression, reflecting HSC activation and transformation, and collagen type I deposition in livers. Immunohistochemistry and rtPCR analysis showed that PGE_2 treatment profoundly inhibited the CCl_4-induced increase in intrahepatic αSMA -, collagen I - and vimentin expression *in vivo* (fig.2a,b,d) Also the signaling pathway of TGFβ was inhibited by PGE_2, as indicated by a significant reduction in pSMAD expression levels in PGE_2-treated

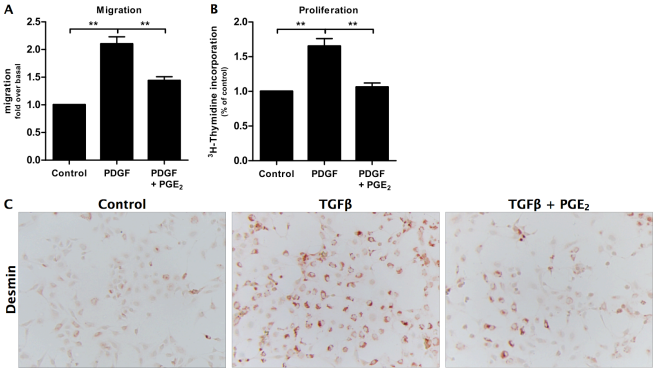

Figure 1. Effect of PGE_2 on fibrosis-related parameters *in vitro*. The effect of PGE_2 on (**A**) PGDF-induced migration and (**B**) proliferation in isolated rat hepatic stellate cells. (**C**) TGFβ-induced collagen-I synthesis in human stellate cells. Bars represents mean ± SEM of 3 experiments performed in duplo, $^{**}P < 0.01$.

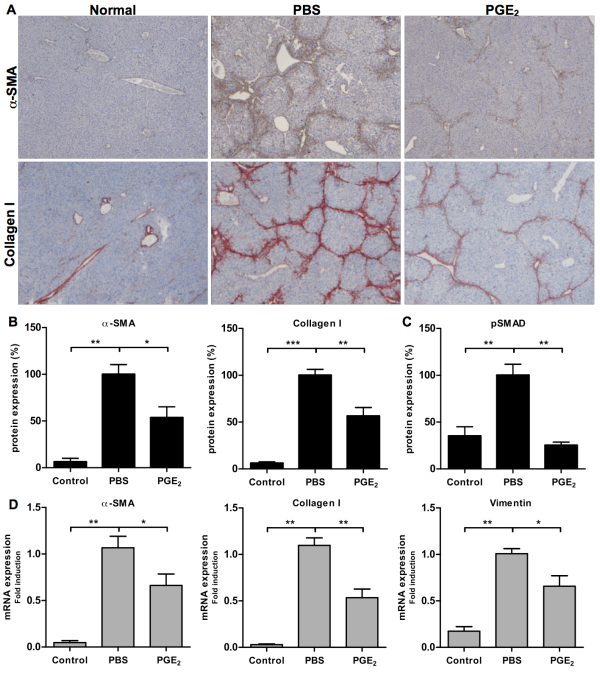

Figure 2. Effect of PGE_2 on fibrosis-related parameters *in vivo*. (**A**) Representative pictures and (**B**) quantitative analysis of α-SMA and collagen-I stained liver sections of normal mice (control) and fibrotic mice treated with PBS or PGE_2. Magnification 40 x. For quantitative analysis, groups were normalized to vehicle group (PBS-treated CCl_4 mice). Figure **C** shows the pSMAD protein expression as analyzed by western blot. (**D**) mRNA expression levels for α-SMA, collagen-I and vimentin.
Bars represents mean ± SEM of 8-9 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$.

fibrotic mice as analyzed by western blot (fig.2c). There were no differences in cytochrome P450 expression (data not shown) as measured by rtPCR, in treated versus untreated fibrotic mice, indicating that PGE_2 did not directly interfere with CCl_4-induced toxicity in hepatocytes.

PGE_2 treatment enhances Epac-1 expression in fibrotic livers and down regulates Rho- activity *in vivo*

It has been reported that PGE_2 exerts anti-fibrotic effects in myofibroblasts *in vitro* through activation of cAMP after binding to its EP2/EP4 receptor[9,18,19]. We therefore investigated the expression levels of the cAMP mediators PKA as well as the recently described Epac-1 pathway *in vivo*. In fibrotic livers a significant reduction in Epac-1 protein expression was observed as compared to normal mice livers. Interestingly, these reduced Epac-1 levels were completely normalized after PGE_2 treatment (fig.3a). In contrast to this , western blot analysis did not show any significant change in PKA expression levels in CCl_4 animals as compared to normal mice. Expression of PKA was also not affected by PGE_2 treatment (fig.3b). No expression of Epac-2 was found in any of the livers.

One of the effectors of Epac-1 is suggested to be Rho kinase, which is an enzyme that is tightly involved in the regulation of cell morphology and fibroblast-to-myofibroblast transdifferentation[14,20,21]. Indeed, immunohistochemical stainings showed a significant upregulation of its downstream mediator phospho-Myosin Light Chain (pMLC) in the fibrotic extracellular matrix bands in CCl_4-treated animals compared to healthy animals. This increase was completely abrogated in PGE_2-treated mice (fig.3c). Collectively, it is shown that PGE_2 attenuates fibrogenesis *in vivo* and this is associated with an increase in Epac-1 levels and inhibition of a putative downstream mediator Rho-kinase, and not with alterations in the PKA levels within the fibrotic livers.

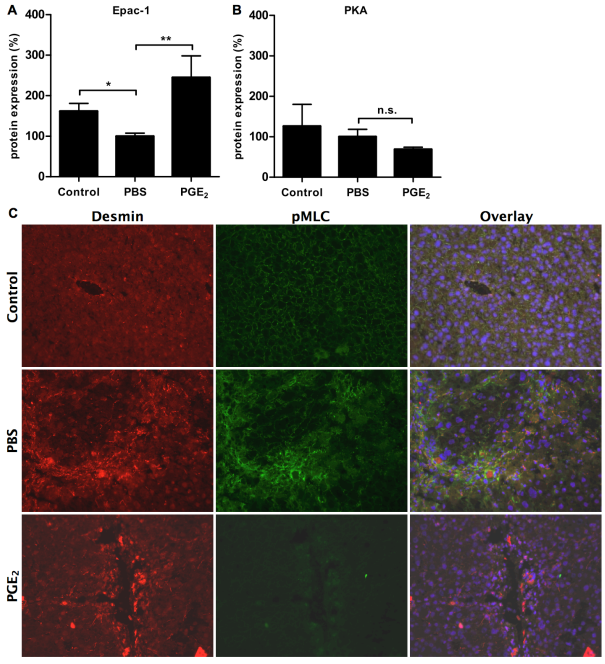

Figure 3. Effect of PGE_2 on intrahepatic Epac-1 expression and Rho-kinase activity *in vivo*. Hepatic protein levels for the cAMP effectors (**A**) Epac-1 and (**B**) PKA as analyzed by western blot. (**C**) Representative pictures of immunofluorescent double staining for phosphorylated-myosin light chain (pMLC, Rho-kinase marker, green) and desmin (HSC marker, red) . Magnification 200 x. Bars represents mean ± SEM of 8-9 mice per group. $*P < 0.05$, $**P < 0.01$.

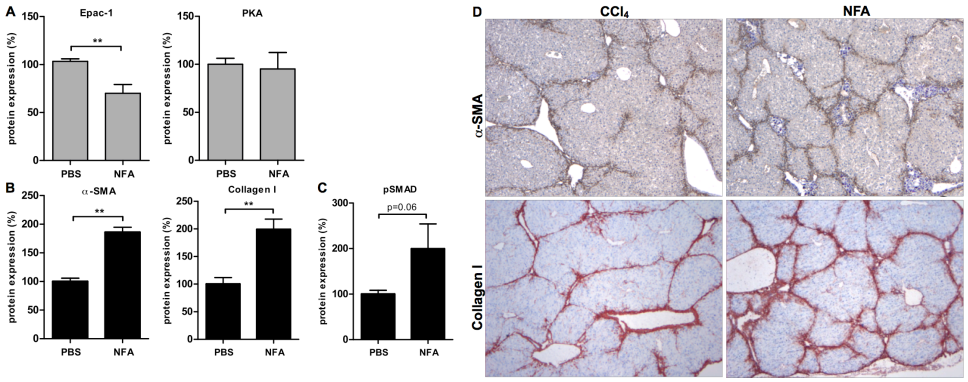

Figure 4. Effect of COX-2 inhibitor Niflumic acid on fibrosis-related parameters *in vivo*. (**A**) Hepatic protein levels for Epac-1 and PKA in mice after treatment with the COX-2 inhibitor Niflumic acid (NFA) as analyzed by western blot, depict a significant reduction in the intrahepatic levels for of Epac-1 by NFA. (**B**) Quantitative analysis and (**D**) representative pictures and of α-SMA and collagen-I stained liver sections of fibrotic mice treated with vehicle (PBS) or NFA. Magnification 40 x. Figure **C** shows the western blot analysis of intrahepatic pSMAD protein levels. Bars represents mean ± SEM of 6 mice per group. $^{**}P < 0.01$.

Effects of the COX-2 inhibitor niflumic acid on Epac-1 expression and fibrogenesis *in vivo*.

If PGE_2 affects the fibrogenic process via Epac-1 expression, then cyclooxygenase inhibitors might also affect these parameters. We therefore treated mice with CCl_4-induced liver fibrosis with the specific COX-2 inhibitor Niflumic acid (n=6) and examined Epac-1 expression and fibrogenesis. In fibrotic mice livers we found that administration of this COX-2 inhibitor was associated with a further decline in intrahepatic Epac-1 expression levels (fig.4a). This was in turn associated with a significant increase of αSMA, collagen-I and p-SMAD expression (fig.4b-d), confirming the earlier observed relationship between Epac-1 and fibrogenesis. Our data indicate that inhibition of endogenous prostaglandin by COX-2 inhibitors leads to pro-fibrotic effects via this pathway.

Epac-1 and PKA reduces HSC activation.

We subsequently used a specific PKA agonist[22,23] and a specific Epac-1 agonist[22,23] to study the involvement of the Epac-1 signalling pathway in myofibroblast activation. Immunohistochemical staining of human LX2 cells also showed a downregulation of TGFβ-induced collagen-1 expression after treatment with the Epac-1 agonist or the PKA agonist (fig.5a) confirming the existence of this pathway in human cells.

Epac-1 expression is impaired in human cirrhotic livers.

We furthermore examined human tissue samples to assess whether Epac-1 expression was also changed in patients with liver diseases of different etiologies. Human samples were obtained from patients suffering from alcohol-induced liver disease, acute liver failure, Wilson's disease, congenital cirrhosis, primary sclerosing cholangitis with intrahepatic cholangiocarcinoma and auto-immune induced hepatitis (n=6). Normal liver tissue samples from patients undergoing partial hepatectomy due to malignancies, served as control (n=6). Analysis of Western blots revealed that Epac-1 expression levels were significantly reduced in all fibrotic livers, irrespective of etiology, compared to control livers (fig.5b).

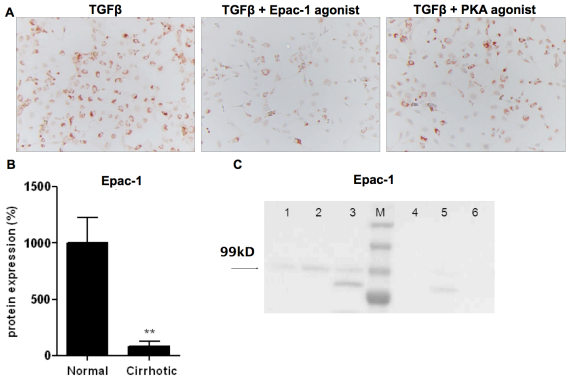

Figure 5. Epac-1 expression in human cirrhotic livers. (**A**) The effect of either Epac-1 or PKA activation by respectively the EPAC-1 agonist 8-pCPT-2'-O-Me-cAMP or the PKA agonist 6-Bnz-cAMP on TGFβ-induced collagen-I synthesis in human stellate cells. (**B**) Quantitative analysis and representative western blot picture of the intrahepatic Epac-1 expression levels in normal (lane 1-3) and fibrotic (lane 4-6) human livers. Bars represents mean ± SEM of 6 human livers per group. ***P* < 0.01. M=marker lane.

DISCUSSION

This study demonstrates the involvement of the novel cAMP effector Epac-1 in the regulation of hepatic fibrogenesis *in vitro* and *in vivo*. Epac proteins, alone or in concert with PKA, are closely involved in the regulation of several pivotal processes including cell survival, cell proliferation and differentiation, immune responses, and cell signaling cascades[14-16]. Despite this effect on cellular key functions, little is known about the role of Epac proteins during liver fibrosis *in vivo*. In cell cultures of fibroblasts of different origin, Yokoyama et al. showed a down regulation of Epac-1 mRNA levels in response to the mitogenic factor TGFβ[16] which may suggest that Epac-1 plays a protective role during fibrogenesis. Our *in vitro* studies in rat HSC and human myofibroblasts and *in vivo* experiments in a chronic CCl_4 mouse model for liver fibrosis now also show an important role for Epac-1 during fibrogenesis. For the first time, profound changes in Epac-1 expression levels during fibrogenesis were found and modification of its expression by PGE_2 and COX-2 inhibitors *in vivo,* was found to affect the fibrotic process significantly.

In vitro, PGE_2 exerts anti-proliferative and anti-fibrogenic effects in fibroblasts through cAMP activation[5,7,8,18,24]. Although PGE_2 is well-known for its role in inflammation and cyclooxygenase products are known to play a role in many diseases including liver fibrosis, the mechanism behind their effects on fibrogenesis has never been extensively studied *in vivo*. A very few publications report on the intrahepatic effects of PGE_2 *in vivo*, i.e. on the accumulation of triglycerides in hepatocytes induced by PGE_2 during ethanol-induced steatosis[25], the inhibitory effect of PGE_2 on the acute phase response[26] and the reduced collagen deposition after treatment with a stable PGE_2 analogue[10], but there is no general concept on its role during liver fibrosis. This lack of clarity *in vivo* is most likely due to its pleiotropic effects and its poor pharmacokinetic profile; it is rapidly cleared by the liver and kidneys, inactivated by plasma proteins or metabolized and oxidized in plasma[11].

Since PGE_2 is a well-known activator of cAMP, we decided to use PGE_2 as an endogenous mediator to examine the role of Epac-1 during liver fibrosis. Our study showed that fibrotic mice treated with PGE_2 developed less scar tissue formation, pSMAD formation and HSC activation within the livers compared to untreated mice.

For the first time, we showed the effects of PGE_2 on Epac-1 levels *in vivo* and the anti-fibrotic effects associated with upregulation of Epac-1 activity. Our data also indicate that the anti-fibrotic effects of

PGE_2 in mouse livers are mediated via cAMP-effector Epac-1, and not via PKA. Mice with advanced liver fibrosis expressed significant lower amounts of hepatic Epac-1 protein compared to normal mice. These levels were normalized by treating the mice with PGE_2 whereas PKA levels did not change significantly during disease progression or after PGE2 treatment. Changes in Epac-1 were paralleled by changes in fibrogenesis within the liver. Our findings are in line with recent studies suggesting that Epac-1 is an endogenous inhibitor of the pro-fibrotic Rho-kinase[14,21]. In the liver, Rho-kinase regulates the transdifferentiation of HSC into the collagen-producing myofibroblastic cells via phosphorylation of its downstream mediators, such as myosin light chain (MLC)[20,27]. Phosphorylated myosin light chain (pMLC) was abundantly expressed in the fibrotic areas of the liver. In fibrotic mice, PGE_2 treatment abolished this formation of pMLC proteins which indicates that the anti-fibrotic effect of PGE_2 is mediated by inhibition of Rho-activity. Our data are in agreement with the notion that inhibition of Rho-kinase activity reduces fibrogenesis[3,20,28] and we now show that PGE_2 affects this activity.

If endogenous PGE_2 is anti-fibrotic via Epac-1 in HSC then the widely used COX inhibitors might also affect this pathway. In our *in vivo* studies we found that inhibition of endogenous PGE_2 production by COX-2 inhibitor Niflumic acid reduced Epac-1 expression levels which was paralleled by a significant increase in scar tissue formation.

Up to now, the pleiotrophc effects of COX activity during liver fibrosis are not well understood. There are contradictory reports in the literature presenting either pro-[29,30] or anti-fibrotic effects[31-33] of COX inhibitors. These conflicting results may indicate a dual effect of these inhibitors on fibrogenesis; their anti-inflammatory effects may account for the anti-fibrogenic results seen after administration of the COX inhibitors during the inflammatory phase of the disease[32-34], whereas their inhibitory effects on local PGE_2 production, or on other cyclooxygenase products in a later stage of disease, may account for their pro-fibrotic effects. Unravelment of the signaling pathway of PGE_2 *in vivo* is therefore essential to understand the exact role of PGE_2 and COX inhibition during disease progression. Our experiments provide an important insight in this. A limited survey on human tissue samples, revealing a reduced Epac-1 expression in human cirrhotic livers (fig 5b) shows that this is also relevant for the human situation. A further decline of Epac-1 induced by COX-inhibitors, as found in our mice studies, may promote the fibrotic process in man as well.

In summary, our study shows the involvement of the novel cAMP effector Epac-1 in the regulation of fibrogenic processes. Fibrogenesis was associated with a reduced Epac-1 expression in mice and human. Treatment of fibrotic mice with the cAMP-activator PGE_2 reversed this and significantly attenuated Rho-activity, collagen deposition and other markers of fibrosis. Moreover, it is shown that COX-2 inhibition affects collagen deposition which was again associated with changes in Epac-1 signalling pathway. Detailed insight in this process is essential to understand the long term effects of COX inhibitors during fibrogenesis, which is relevant to millions of people world-wide.

ACKNOWLEDGEMENTS

The technical assistance of Mrs. Catharina Reker-Smit is greatly appreciated.

REFERENCES

1. Pinzani M, Rombouts K, Colagrande S. Fibrosis in chronic liver diseases: Diagnosis and management. *J Hepatol*. 2005;42 Suppl(1):S22-36.
2. Heymann F, Trautwein C, Tacke F. Monocytes and macrophages as cellular targets in liver fibrosis. *Inflamm Allergy Drug Targets*. 2009;8(4):307-318.
3. Friedman SL. Liver fibrosis -- from bench to bedside. *J Hepatol*. 2003;38 Suppl 1:S38-53.
4. Schuppan D, Afdhal NH. Liver cirrhosis. *Lancet*. 2008;371(9615):838-851.
5. Mallat A, Gallois C, Tao J, et al. Platelet-derived growth factor-BB and thrombin generate positive and negative signals for human hepatic stellate cell proliferation. role of a prostaglandin/cyclic AMP pathway and cross-talk with endothelin receptors. *J Biol Chem*. 1998;273(42):27300-27305.
6. Swaney JS, Roth DM, Olson ER, Naugle JE, Meszaros JG, Insel PA. Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. *Proc Natl Acad Sci U S A*. 2005;102(2):437-442.
7. Haag S, Warnken M, Juergens UR, Racke K. Role of Epac1 in mediating anti-proliferative effects of prostanoid EP(2) receptors and cAMP in human lung fibroblasts. *Naunyn Schmiedebergs Arch Pharmacol*. 2008;378(6):617-630.
8. Weinberg E, Zeldich E, Weinreb MM, Moses O, Nemcovsky C, Weinreb M. Prostaglandin E2 inhibits the proliferation of human gingival fibroblasts via the EP2 receptor and epac. *J Cell Biochem*. 2009;108(1):207-215.
9. Huang S, Wettlaufer SH, Hogaboam C, Aronoff DM, Peters-Golden M. Prostaglandin E(2) inhibits collagen expression and proliferation in patient-derived normal lung fibroblasts via E prostanoid 2 receptor and cAMP signaling. *Am J Physiol Lung Cell Mol Physiol*. 2007;292(2):L405-13.
10. Ruwart MJ, Wilkinson KF, Rush BD, et al. The integrated value of serum procollagen III peptide over time predicts hepatic hydroxyproline content and stainable collagen in a model of dietary cirrhosis in the rat. *Hepatology*. 1989;10(5):801-806.
11. Hamberg M, Samuelsson B. On the metabolism of prostaglandins E 1 and E 2 in man. *J Biol Chem*. 1971;246(22):6713-6721.
12. Bezugla Y, Kolada A, Kamionka S, Bernard B, Scheibe R, Dieter P. COX-1 and COX-2 contribute differentially to the LPS-induced release of PGE2 and TxA2 in liver macrophages. *Prostaglandins Other Lipid Mediat*. 2006;79(1-2):93-100.
13. de Rooij J, Zwartkruis FJ, Verheijen MH, et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*. 1998;396(6710):474-477.
14. Roscioni SS, Elzinga CR, Schmidt M. Epac: Effectors and biological functions. *Naunyn Schmiedebergs Arch Pharmacol*. 2008;377(4-6):345-357.
15. Grandoch M, Roscioni SS, Schmidt M. The role of epac proteins, novel cAMP mediators, in the regulation of immune, lung and neuronal function. *Br J Pharmacol*. 2010;159(2):265-284.
16. Yokoyama U, Patel HH, Lai NC, Aroonsakool N, Roth DM, Insel PA. The cyclic AMP effector epac integrates pro- and anti-fibrotic signals. *Proc Natl Acad Sci U S A*. 2008;105(17):6386-6391.
17. Geerts A, Niki T, Hellemans K, et al. Purification of rat hepatic stellate cells by side scatter-activated cell sorting. *Hepatology*. 1998;27(2):590-598.
18. Huang SK, Wettlaufer SH, Chung J, Peters-Golden M. Prostaglandin E2 inhibits specific lung fibroblast functions via selective actions of PKA and epac-1. *Am J Respir Cell Mol Biol*. 2008;39(4):482-489.
19. Kojima F, Kapoor M, Kawai S, Yang L, Aronoff DM, Crofford LJ. Prostaglandin E2 activates Rap1 via EP2/EP4 receptors and cAMP-signaling in rheumatoid synovial fibroblasts: Involvement of Epac1 and PKA. *Prostaglandins Other Lipid Mediat*. 2009;89(1-2):26-33.
20. Mack CP, Somlyo AV, Hautmann M, Somlyo AP, Owens GK. Smooth muscle differentiation marker gene expression is regulated by RhoA-mediated actin polymerization. *J Biol Chem*. 2001;276(1):341-347.
21. Zieba BJ, Artamonov MV, Jin L, et al. The cAMP-responsive Rap1 guanine nucleotide exchange factor, epac, induces smooth muscle relaxation by down-regulation of RhoA activity. *J Biol Chem*. 2011;286(19):16681-16692.

22. Roscioni SS, Maarsingh H, Elzinga CR, et al. Epac as a novel effector of airway smooth muscle relaxation. *J Cell Mol Med*. 2011;15(7):1551-1563.

23. Roscioni SS, Kistemaker LE, Menzen MH, et al. PKA and epac cooperate to augment bradykinin-induced interleukin-8 release from human airway smooth muscle cells. *Respir Res*. 2009;10:88.

24. Mallat A, Preaux AM, Serradeil-Le Gal C, et al. Growth inhibitory properties of endothelin-1 in activated human hepatic stellate cells: A cyclic adenosine monophosphate-mediated pathway. inhibition of both extracellular signal-regulated kinase and c-jun kinase and upregulation of endothelin B receptors. *J Clin Invest*. 1996;98(12):2771-2778.

25. Enomoto N, Ikejima K, Yamashina S, et al. Kupffer cell-derived prostaglandin E(2) is involved in alcohol-induced fat accumulation in rat liver. *Am J Physiol Gastrointest Liver Physiol*. 2000;279(1):G100-6.

26. Rincon-Sanchez AR, Covarrubias A, Rivas-Estilla AM, et al. PGE2 alleviates kidney and liver damage, decreases plasma renin activity and acute phase response in cirrhotic rats with acute liver damage. *Exp Toxicol Pathol*. 2005;56(4-5):291-303.

27. Pellegrin S, Mellor H. Actin stress fibres. *J Cell Sci*. 2007;120(Pt 20):3491-3499.

28. Fukushima M, Nakamuta M, Kohjima M, et al. Fasudil hydrochloride hydrate, a rho-kinase (ROCK) inhibitor, suppresses collagen production and enhances collagenase activity in hepatic stellate cells. *Liver Int*. 2005;25(4):829-838.

29. Mohammed NA, Abd El-Aleem SA, El-Hafiz HA, McMahon RF. Distribution of constitutive (COX-1) and inducible (COX-2) cyclooxygenase in postviral human liver cirrhosis: A possible role for COX-2 in the pathogenesis of liver cirrhosis. *J Clin Pathol*. 2004;57(4):350-354.

30. Reilly TP, Brady JN, Marchick MR, et al. A protective role for cyclooxygenase-2 in drug-induced liver injury in mice. *Chem Res Toxicol*. 2001;14(12):1620-1628.

31. Cheng J, Imanishi H, Liu W, et al. Inhibition of the expression of alpha-smooth muscle actin in human hepatic stellate cell line, LI90, by a selective cyclooxygenase 2 inhibitor, NS-398. *Biochem Biophys Res Commun*. 2002;297(5):1128-1134.

32. Paik YH, Kim JK, Lee JI, et al. Celecoxib induces hepatic stellate cell apoptosis through inhibition of akt activation and suppresses hepatic fibrosis in rats. *Gut*. 2009;58(11):1517-1527.

33. Chavez E, Segovia J, Shibayama M, et al. Antifibrotic and fibrolytic properties of celecoxib in liver damage induced by carbon tetrachloride in the rat. *Liver Int*. 2010;30(7):969-978.

34. Denda A, Endoh T, Kitayama W, et al. Inhibition by piroxicam of oxidative DNA damage, liver cirrhosis and development of enzyme-altered nodules caused by a choline-deficient, L-amino acid-defined diet in rats. *Carcinogenesis*. 1997;18(10):1921-1930.

CHAPTER 5

The hepatic stellate cell is the key effector cell in PGE_2-induced changes in Epac-1.

Marlies Schippers, Eduard Post, Leonie Beljaars, Klaas Poelstra

Department of Pharmacokinetics, Toxicology and Targeting, University of Groningen, The Netherlands

ABSTRACT

Background & Aimes: Prostaglandin E_2 (PGE_2) is a lipid modulator of many (patho) physiological processes in the body including inflammation and fibrogenesis. It affects cAMP activity by binding to its G-coupled EP receptors, presented on a variety of cells, but its mechanism of action is unclear. cAMP mediator, Exchange protein activated by cAMP-1 (Epac-1), was recently discovered as a new signaling mediator during fibrogenesis. We now aim to study the mechanism of action of PGE_2 on fibrogenesis by examining its effect on Epac-1 expression. In this study we also investigated which cell type in the liver is the effector cell of the PGE_2-mediated effects *in vivo* by using a cell-specific drug delivery approach.

Methods: Liver fibrosis was induced by administration of CCl_4 to Balb/c mice for 8 weeks. In week 7 and 8, animals received either vehicle or PGE_2-conjugates that deliver PGE_2 selectively to either hepatocytes, Kupffer cells (KC) or hepatic stellte cells (HSC) (n = 6-9 per group). Results: Selective delivery of PGE_2 to hepatocytes had no effect on collagen deposition and intrahepatic Epac-1 and PKA expression. KC-directed PGE_2 induced higher scar tissue formation, which was independent of PKA and Epac-1 activities. However, in HSC, PGE_2 treatment induced lower collagen expression levels which was associated with higher intrahepatic Epac-1 expression levels and lower Rho-kinase activity.

Conclusions: This study provides insight into the antifibrotic effect of PGE_2 during liver fibrogenesis and pinpoints the hepatic stellate cell as a key target cell for this prostaglandin-induced effect. Moreover, using cell-specific delivery approaches we identify Epac-1 as a major intracellular signaling pathway for these PGE_2-mediated anti-fibrotic effects in HSC *in vivo*.

INTRODUCTION

The endogenous mediator Prostaglandin E_2 (PGE_2) is derived from the oxidation of arachidonic acid by cyclooxygenase-2 and is involved in the modulation of different (patho) physiological processes such as inflammation, fibrosis and cancer, in various organs. The diverse effects of PGE_2 can be ascribed to the fact that PGE_2 acts via its G-coupled protein receptors EP1 to EP4 which are present on multiple cell types and affect the second messenger cyclic AMP (cAMP) in different ways[1].

In the liver, PGE_2 is known to regulate glycogenolysis in hepatocytes[2,3], the release of inflammatory mediators in Kupffer cells (KC)[4,5] and activation of hepatic stellate cells (HSC)[6-8]. During chronic injury in the liver by i.e. toxins, drugs, lipid particles or alcohol, damaged hepatocytes secrete various inflammatory cytokines that activate KC and HSC which finally may result in liver fibrogenesis. This chronic disease is an uncontrolled wound healing process, characterized by an abundant deposition of extracellular matrix (ECM) produced by HSC[9,10]. Activated macrophages also stimulate wound healing by releasing growth factors that further activate HSC[11-13]. HSC activation and differentiation is tightly controlled by cAMP activity. In fibroblasts, PGE_2 exerts anti-fibrotic activities through cAMP activation by binding to its EP_2 or EP_4 receptor[6,14-17]. *In vivo*, treatment of rats with a stable PGE_2 analogue resulted in a reduced collagen deposition[18] but the mechanism behind this was never revealed.

Next to the classic cAMP effector Protein Kinase A (PKA), Exchange Protein Activated by cAMP (Epac) was recently discovered as a new signaling mechanism for cAMP-mediated effects[19]. Epac is involved in the regulation of several cellular key processes such as calcium handling, neural signalling, inflammation, cell proliferation and migration, by promoting the exchange of GTP and GDP in the GTPase cycle[20,21]. A reduced Epac-1 expression by TGFβ was recently found in cultures of hepatic stellate cells, suggesting a role for this pathway during fibrogenesis[22]. In addition, in cultured fibroblasts, PGE_2 treatment was found to influence cAMP effectors PKA and Epac[23,24], again suggesting a role for Epac signaling in the fibrogenic process. Since fibrogenesis is a multi-cellular process and Epac-1 proteins are also expressed in several cell types including monocytes, macrophages, B and T cells and blood cells[21] we now aim to investigate which cell type in the liver is the effector cell of the PGE_2-mediated effects *in vivo* and the relevance of Epac-1 in PGE_2-mediated effects. We used a cell-specific drug delivery approach to identify the effector cells and signaling pathways that mediate these PGE_2-induced effects *in vivo*. PGE_2 was coupled to cell-specific carriers directed at hepatic stellate cells, Kupffer cells or hepatocytes and we subsequently evaluated fibrogenic parameters and PKA and Epac-1 expression levels in a chronic CCl_4-induced mice model for liver fibrosis.

This study provides insight into the effect of PGE_2 on ECM deposition and pinpoints the hepatic stellate cell as a key target cell for this prostaglandin-induced effect. Moreover, we identify Epac-1 as an important intracellular signalling pathway for PGE_2-mediated anti-fibrotic effects in HSC *in vivo*.

MATERIAL & METHODS

Materials

The following primary antibodies were used: rabbit anti-CYP2E1 (Millipore Corporation, Darmstadt, Germany), goat anti-HSA (Cappel, USA), goat anti-Desmin (Santa Cruz Biotecnology, Heidelberg, Ger-

many), rat anti-CD68 (AbD Serotec, Dusseldorf, Germany), goat anti-collagen I (Southern Biotechnology Associates, Birmingham, AL), goat anti-Epac-1 (Santa Cruz Biotecnology, Heidelberg, Germany), goat anti-PKA mouse and anti-pMLC2 (Ser19) (Cell Signaling Technology, Danvers, MA). Species-specific horseradish peroxidase and FITC-/TRITC-conjugated secondary antibodies were purchased from Dako Denmark A/S (Glostrup, Denmark).

Conjugates

Synthesis of pPBHSA, manHSA and lac-HSA: PDGFβ-receptor recognizing peptides C*SRNLIDC* (pPB, Ansynth Service BV, Roosendaal, The Netherlands), mannose (p-aminophenyl-a-D-mannopyranoside, Sigma-Aldrich, St. Louis, MO), and chemically pure lactose (Lamers & Lindemans BV, The Netherlands), were covalently coupled to Human Serum Albumin as described previously[25,26].

Conjugation of PGE_2 to modified Albumin: PGE_2 (Cayman Chemicals, Ann Arbor, MI) was dissolved in dried dimethylformamid (10 mg/ml). The carboxylic acid group of PGE_2 was activated with N,N-dicyclohexylcarbodiimide (DCC), and N-hydroxysuccinimid (NHS). This PGE_2/DCC/NHS solution was reacted overnight at room temperature with pPBHSA, manHSA or lacHSA (10 mg/ml). The reaction product containing PGE_2-conjugates were subsequently dialyzed against PBS using a 10 KDa dialysis cassette (Slide-A-Lyzer 10K, Pierce Biotechnology, Rockford, IL, USA), and further purified with FPLC. Conjugates were lyophilized and stored at −20°C. The amount of PGE_2 coupled to the carrier was assessed by mass spectrometry. The concentrations of the PGE_2-carrier constructs and the dose of each carrier construct *in vivo* was adjusted to the amount of PGE_2 coupled to the protein: all experiments with all carriers were performed with equimolar PGE_2 concentrations.

Cell experiments

Primary HSC were isolated from livers of Wistar rats as previously described (Geerts et al. 1998). After isolation, HSC were cultured on plastic for 10 days to obtain an activated phenotype before experiments. Human HSC (LX2) were kindly provided by Prof. S.L. Friedman (Mount Sinai Hospital, New York). LX2 were cultured in DMEM-Glutamax (Invitrogen) supplemented with 10% FBS. All data are presented as the mean of three different experiments performed in duplo.

Proliferation Assay: Primary HSC were seeded in 12-wells plates and grown for 2 days. After starvation, cells were incubated with 5 μM PGE_2, 50 μM Epac-1 agonist (8-pCPT-2'-O-Me-cAMP: Biolog, Bremen, Germany) or 500 μM PKA agonist (6-Bnz-cAMP; Biolog) along with 50 ng/ml PDGF-BB (PeproTech, Rocky Hill, NJ). After 18h, 3H-thymidin (final concentration 0.25 uCi/ml) was added for 6h. Cells were fixed with 5% TCA and lysed with 1M NaOH. Proliferation was counted using a scintillation counter (Perkin Elmer).

Rat HSC Migration Assay: Primary HSC were added to Transwell chambers, with 5 μM PGE_2, 50 μM Epac-1 agonist, or 500 μM PKA agonist. PDGF-BB (50 ng/ml) was added to the other chamber to stimulate migration. Cells were incubated for 24h, fixed, and stained with hematoxylin. Cells on both sides of the membrane were counted in five fields/membrane at 40x magnification. Migration was calculated as the percentage of cells at the lower side of the membrane relative to the total number of cells in each field.

LX2 Collagen-I deposition: LX2 cells were starved (24h) and incubated with 5 μM PGE_2 along with 5 ng/ml TGFβ (Roche Diagnostics, Mannheim, Germany), for 24h. Cells were then fixed and stained for collagen using goat anti-Collagen I IgG and standard indirect immunoperoxidase methods.

Animal Experiments

All animal experiments were approved by the Animal Ethics Committee of the University of Groningen, the Netherlands. Animals were purchased from Harlan (Zeist, Netherlands).

CCl_4-Induced Liver Fibrosis Model: Male balb/c mice (20-22 g) were injected twice a week intraperitoneally with PBS or increasing doses of CCl_4 in olive oil (week 1, 0.5 ml/kg; week 2, 0.8 ml/kg and week 3-8, 1 ml/kg). At week 7 and 8, mice were treated intravenously with vehicle (PBS), PGE_2-pPBHSA, pPBHSA, PGE_2-manHSA, manHSA, PGE_2-lacHSA, or lacHSA (equivalent to 0.5 mg/kg PGE_2, three times per week n = 6 per group. Mice were sacrificed at week 8.

Immunohistochemistry and quantitative analysis of sections: Immunohistochemistry was performed on 4-μm cryostat sections or cells according to standard methods. Immunofluorescent staining in sections was visualized using a M.O.M. kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Immunohistochemical stainings were quantitated by analysing complete sections from 3 different liver lobes of each animal at magnification 10x10 using the Cell D image analysing software (Olympus, Hamburg, Germany).

Western Blot Analysis: Liver homogenates were subjected to SDS-PAGE (12%) and proteins were transferred to PVDF membrane according to standard methods. Blots were developed using Western Lightning-ECL reagent (Perkin-Elmer, Boston, MA) according to the manufacturer's instructions and quantified with Genetools (Syngene, Cambridge, UK). Of each animal, samples from 3 different liver lobes were analyzed.

Quantitative Real Time PCR: Total RNA from rHSC was isolated from HSC using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA). Total RNA (1.6 ug) was reverse transcripted in 50 μl using cDNA synthesis kit (Invitrogen, Carlsbad, CA). The primers of Epac-1 (forward: CAGTGCTGCTCTGGCCGGGA, reverse: GTTCCTGCAGGCTGGGGCTC), PKA (forward: GGTTCAGTGAGCCCCACGCC, reverse: GGGGGTCCCACACAAGGTCCA) and β-actin (forward: GGCATCCTGACCCTGAAGTA, reverse: GGGGTGTTGAAGGTCTCAAA) for real-time quantitative PCR were purchased from Sigma Genosys (Haverhill, UK). The reactions were analyzed by ABI7900HT sequence detection system (Applied Biosystems, Foster City, California). The threshold cycles (Ct) were calculated and relative gene expression was analyzed after normalizing for β-actin, house-keeping gene.

Proliferation Assay: To examine the proliferation of primary HSC, cells were seeded in 12-well culture plates (2 x 10^5 cells per well) and grown for 2 days. Cells were starved overnight in serum-free medium and were incubated with 50 μM Epac-1 agonist or 500 μM PKA agonist along with 50 ng/ml PDGF-BB. After 18h, 3H-thymidin (final concentration of 0.25 uCi/ml) was added for 6h. Subsequently, cells were washed with PBS, fixed with TCA 5% and lysed with 1M NaOH. Proliferation of HSC were counted with Optima Gold Scintillation fluid using a scintillation counter (Perkin Elmer).

rHSC Migration Assay: To examine the migration of primary HSC, 6×10^4 cells per well were added to the upper compartment of a Transwell chamber, together with 50 µM Epac-1 agonist or 500 µM PKA agonist. PDGF-BB (50 ng/ml) was added to the lower chamber to stimulate migration. The cells were then incubated for 24 h, fixed, and stained with Mayer's hematoxylin. Cells on both sides of the membrane were counted in five fields per membrane at 40× magnification. Migration was calculated as the percentage of cells on the lower side of the membrane relative to the total number of cells in each field.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using the Mann-Whitney t test. $p < 0.05$ was considered as significant.

RESULTS

Epac-1 expression is reduced during fibrogenesis *in vivo*

In a chronic CCl_4 model of liver fibrosis, a very high deposition of the extracellular matrix component collagen I was observed relative to normal mice (fig.1a). In these fibrotic livers, lower intrahepatic expression levels of cAMP mediator Epac-1 were found compared to normal livers (fig.1b) as measured by western blot methods. We observed a significant correlation between the amount of collagen deposition and Epac-1 expression levels in all these animals (r^2=0.40, p<0.02) (fig.1c). To investigate the causality of the correlation between collagen I and Epac-1, mice with CCl_4-induced fibrosis were treated for 2 weeks with the anti-fibrotic mediator PGE_2. We found that a reduction in collagen deposition in the fibrotic bands induced by PGE_2 was associated with higher intrahepatic Epac-1 expression levels (r^2=0.28, p<0.03) (fig.1d).

We subsequently studied the direct effect of PGE_2 in hepatocytes, KC or HSC, during fibrogenesis. To assess the effect of PGE_2 in different cell types we used a set of cell-specific carriers that can deliver the prostaglandin to each resident hepatic cell type separately.

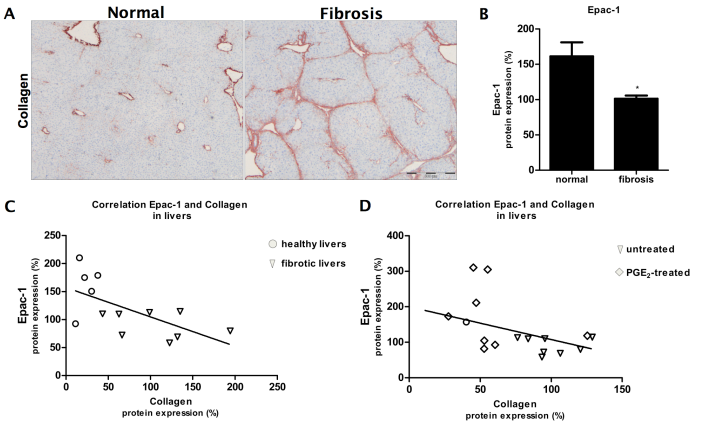

Figure 1. Association Epac-1 and collagen deposition *in vivo*.
(**A**) Representative pictures of collagen-I stained liver sections of normal mice (control) and fibrotic mice, 8 weeks after the onset of CCl_4 treatment. (**B**) Western blot analysis of hepatic Epac-1 protein expression levels and (**C**) the correlation between collagen I deposition and Epac-1 protein expression in livers of normal and fibrotic mice (r^2=0.40, p<0.02). (**D**) Correlation between collagen I and Epac-1 protein expression in fibrotic livers of PGE-treated and untreated mice (r^2=0.28, p<0.03). Bars represents mean ± SEM of 6-9 mice per group. *$P < 0.05$ versus untreated normal mice.

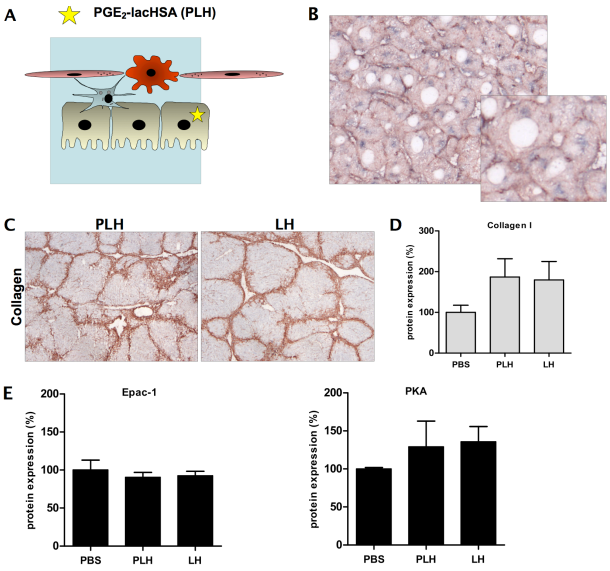

Figure 2. Effect of PGE_2 targeted to hepatocytes on collagen deposition and intrahepatic Epac-1 and PKA expression *in vivo*. Targeting to hepatocytes: (**A**) schematic overview and (**B**) double staining for the hepatocyte plasma membrane marker (CYP; red) and human serum albumin (blue). (**C**) Representative pictures and (**D**) western blot quantification of collagen I staining in fibrotic livers of mice treated with vehicle (PBS), PGE_2-lacHSA (PLH) or carrier (LH) alone. (**E**) Hepatic protein levels for the cAMP effectors Epac-1 and PKA as analyzed by western blot.

Specific delivery of PGE_2 to hepatocytes has no effect on collagen deposition and hepatic Epac-1 and PKA expression

The asialoglycoprotein receptor on hepatocytes has been widely used for cell-specific delivery of compounds to these cells[27]. Lactosylated albumin (LacHSA) is a ligand for this receptor[25], and we therefore coupled PGE_2 to this modified albumin to achieve cell-specific uptake of PGE_2 in hepatocytes (fig.2a). PGE_2-lacHSA (PLH) conjugate yielded a pharmacologically active conjugate in cell cultures (data not shown) and immunohistochemical staining showed co- localization of HSA (blue) and hepatocyte marker CYP2E1(red) (fig.2b). Mass spectrometry analysis revealed that 8 molecules PGE_2 were coupled per albumin molecule. Immunohistochemical staining and western blot analysis showed no differences in collagen I levels in mouse livers after treatment with PHL or the carrier alone (fig.2c,d). Subsequently, we studied the Epac-1 and PKA expression levels in the fibrotic livers of mice treated with our conjugate and their respective control groups by western blot analysis. The intrahepatic levels of the cAMP effectors PKA and Epac-1 in the PLH-treated animals were identical to the levels in untreated fibrotic animals or animals treated with the carrier alone (LH) (fig.2e).

Specific delivery of PGE_2 to Kupffer cells induces higher collagen deposition but no changes in cAMP effectors PKA and Epac-1

To deliver PGE_2 to the macrophages we used a carrier in which mannose groups were coupled to human serum albumin (manHSA). The mannose-receptor is abundantly expressed on KC[25,28]. The cell-specificity of this drug carrier also has been described *in vivo* in earlier reports[25,29]. Synthesis of the PGE_2-manHSA (PMH) conjugate (fig.3a) yielded a pharmacologically active conjugate in cell cultures (data not shown) that contained 16 molecules PGE_2 per albumin molecule. Immunohistochemical staining showed co-localization of HSA (red) and macrophage marker CD68 (blue) confirming delivery of this construct to KC (fig.3b). Selective delivery of PGE_2 to Kupffer cells led to a higher collagen I deposition in fibrotic livers as

compared to the vehicle group or the group treated with the carrier alone, analyzed by western blot. The carrier alone had no significant effect (fig. 3c,d). Western blot analysis showed no changes in intrahepatic PKA and Epac-1 protein levels after treatment with PMH as compared to untreated fibrotic mice or mice treated with the carrier alone (MH) (fig.3e).

Specific delivery of PGE_2 to fibroblasts inhibits collagen deposition and enhances hepatic Epac-1 expression

In fibrotic livers, the PDGFβ-receptor is abundantly expressed on activated HSC[30,31]. We therefore coupled a PDGFβ-receptor recognizing cyclic peptide[26] to human serum albumin (PH) and used this carrier to deliver PGE_2 to the activated HSC (fig.4a) The cell-specificity of the PDGF-directed carrier has been demonstrated *in vivo* in previous studies [26,32]. Coupling of PGE_2 to PH also yielded a pharmacologically active compound *in vitro*, and results of mass spectrometry indicated 7 molecules PGE_2 coupled to each albumin molecule. This conjugate accumulated *in vivo* in activated HSC as determined by immunohistochemistry; co-localization of HSA (red) and HSC marker desmin (blue) was observed in the fibrotic areas (fig.4b). In mice treated with the HSC-selective PGE_2 conjugate PPH, but not in mice that received the carrier alone, western blot analysis showed significant lower collagen I deposition levels compared to untreated fibrotic mice (fig 4c,d). In livers of PPH-treated mice, we also found a significant upregulation of Epac-1 expression levels compared to untreated mice or to mice receiving PH alone. No change in PKA expression levels was observed in livers of any of the groups (fig.4e).

Only HSC-specific PGE_2 induces lower Rho-kinase expression levels *in vivo*

One of the downstream effectors of Epac-1 is Rho kinase, which is an enzyme that is tightly involved in the regulation of cell morphology and fibroblast-to-myofibroblast transdifferentiation[33,34]. Rho-kinase activity leads to phosphorylation of Myosin Light Chain (MLC) and pMLC expression can be applied as a marker for Rho kinase activity[35,36]. Significant staining for this pMLC protein was found in fibrotic septa in CCl_4-treated mice. No staining was found in the parenchymal area or hepatocytes. Since macrophages

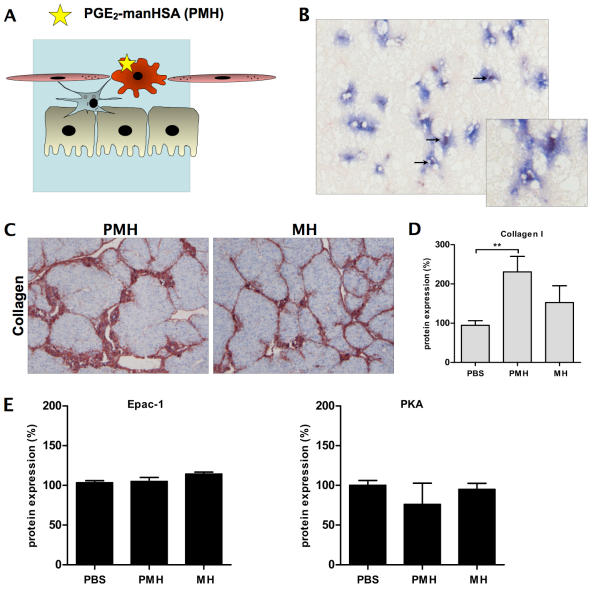

Figure 3. Effect of PGE_2 targeted to Kupffer cells on collagen deposition and intrahepatic Epac-1 and PKA expression *in vivo*. Targeting to Kupffer cells: (**A**) schematic overview and (**B**) double staining for the KC-marker CD68 (red) and human serum albumin (blue). Arrows indicate double-positive cells. (**C**) Representative pictures and (**D**) western blot quantification of collagen I in fibrotic livers of mice treated with vehicle, PGE_2-manHSA (PMH) or carrier alone (MH). (**E**) Hepatic protein levels for the cAMP effectors Epac-1 and PKA as analyzed by western blot. Bars represents mean ± SEM of 6 mice per group. $^{**}P < 0.01$ versus untreated fibrotic mice.

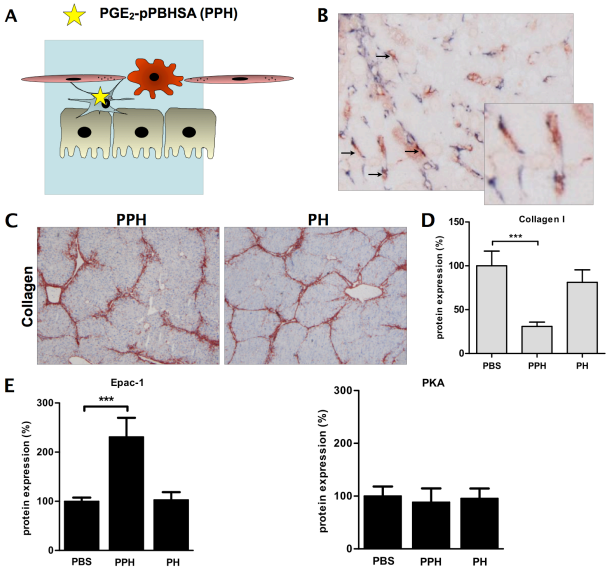

Figure 4. Effect of PGE_2 targeted to HSC on collagen deposition and intrahepatic Epac-1 and PKA expression *in vivo*. Targeting to HSC: (**A**) schematic overview and (**B**) double staining for the HSC-marker desmin (blue) and human serum albumin (red). Arrows indicate double-positive cells. (**C**) Representative pictures and (**D**) western blot quantification of collagen I in fibrotic livers of mice treated with vehicle, PGE_2-pPBHSA (PPH) or carrier alone (PH). (**E**) Hepatic protein levels for the cAMP effectors Epac-1 and PKA as analyzed by western blot. Bars represents mean ± SEM of 8-9 mice per group. ***$P < 0.001$ versus untreated normal mice.

and myofibroblastic-like cells accumulate in these fibrotic septa we examined whether PGE_2 delivery to KC or HSC affected pMLC staining. In mice treated with the HSC-selective PGE_2 conjugate (PPH) we observed significant lower levels of pMLC protein expression (fig.5a), whereas no difference in pMLC protein expression was observed in mice treated with the KC-selective PGE_2 conjugate PMH (fig.5b). No effect was observed in livers of mice treated with one of the carriers alone (MH or PH).

In summary, these drug targeting studies show that the HSC, rather than KC or hepatocytes are the effector cells of PGE_2-mediated anti-fibrotic effects and our data indicate that this effect is mediated by Epac-1 and Rho-kinase activity.

Epac-1 expression in HSC decreases during fibrogenesis

To further explore our *in vivo* data, implicating HSC-specific Epac-1 stimulation by PGE_2, we performed additional *in vitro* studies in primary rHSC. In rHSC, reduced Epac-1 mRNA expression levels were observed after administration of the pro-fibrogenic agents PDGF-BB and TGFβ compared to un-stimulated cells. This is in agreement with the reduced Epac-1 expression levels seen *in vivo* in fibrotic livers (fig.6a). We subsequently used a specific PKA agonist (6-Bnz-cAMP)[37,38] and a specific Epac-1 agonist (8-pCPT-2'-O-Me-cAMP)[37,38] to study the involvement of the Epac-1 signaling pathway in fibrogenic responses of the myofibroblasts. Both Epac-1 and PKA activation induced anti-fibrotic effects in fibroblasts; the Epac-1 agonist significantly inhibited the PDGF-induced migration and proliferation in rHSC whereas the PKA agonist only inhibited PDGF-induced migration significantly (fig.6b). These *in vitro* results confirm the involvement of both Epac-1 and PKA in the pro-fibrotic activity of HSC

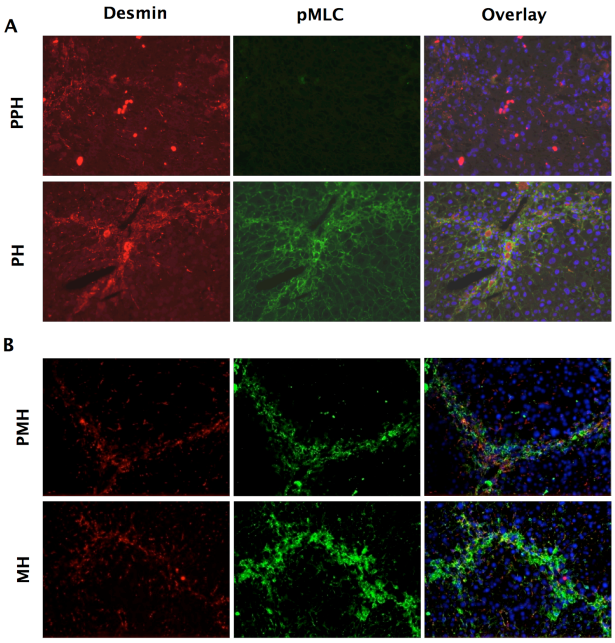

Figure 5. The effect of PGE_2 targeted to HSC (PPH) or to KC (PMH) on Rho-kinase activity *in vivo*. (**A**) Representative pictures of immunofluorescent staining for phosphorylated-myosin light chain (pMLC green) and desmin (red) in fibrotic mice treated with PPH or PH. (**B**) Immunofluorescent staining for pMLC in fibrotic mouse livers treated with PMH, or MH alone.

DISCUSSION

Our study demonstrates that PGE_2 has opposite effects on ECM deposition when it is delivered to different cell types in the liver. Delivery to KC yields higher ECM deposition whereas delivery to HSC leads to reduced scar tissue formation. Only in HSC, PGE_2 affected intrahepatic Epac-1 expression levels significantly. Collectively, our study demonstrates that HSC, and not hepatocytes or KC nor infiltrating inflammatory cells, are responsible for the reduced ECM deposition seen after PGE_2 treatment in the fibrotic liver and that the Epac-1 signaling pathway plays a significant role in this.

In various cell types, PGE_2 activates cAMP signaling via its EP2 and EP4 receptor, but it can also act as a negative effector by inhibition of cAMP signaling via its EP3 receptor, implicating its important regulatory effect in many (patho) physiological processes. Liver fibrosis is the result of chronic inflammation which eventually leads to a deregulated balance between collagen synthesis and degradation[12]. The multiple effects of PGE_2 on immune-cells have been widely studied[39-42] and its effect on inflammation might in turn lead to anti-fibrotic effects. In addition, PGE_2 might also directly affect resident hepatic cells. Hepatocytes, macrophages and fibroblasts are tightly involved in the development of liver fibrosis. Fibroblasts are the main producers of collagen and are activated by pro-and anti-inflammatory cytokines secreted by hepatocytes and macrophages. In fibroblasts of different origin, activation of cAMP via EP2 and EP4 receptors revealed a role for Epac-1 in the proliferation and activation of these cells[6,17,23,24]. In addition, PGE_2 regulates antigen presenting cell functions via EP2 and EP4, and suppresses cytokine production in macrophages via the EP4 receptor[43]. In muscularis resident macrophages, PGE_2 induces iNOS expression via EP2 and EP4[44] and anti-inflammatory actions in alveolar macrophages also via activation of these EP receptors[45]. In hepatocytes EP receptors are involved in the proliferation of these cells[46,47]. *In vivo*, treatment of rats with a stable analogue of PGE_2 induced lower collagen expression levels compared to

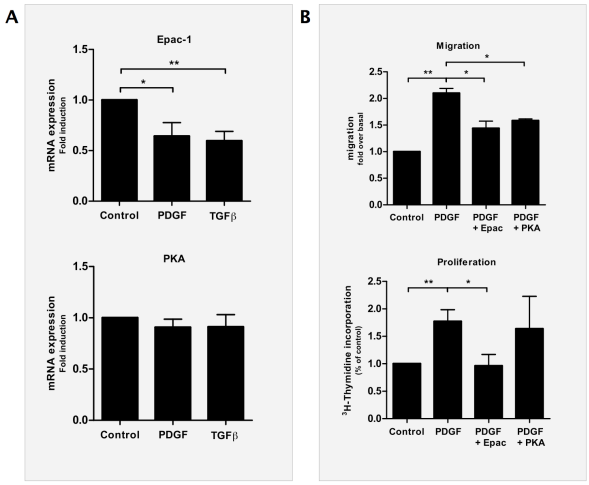

Figure 6. Effect of Epac-1 or PKA activation by specific agonists *in vitro*. (**A**) Quantitative real-time PCR analysis of Epac-1 and PKA mRNA expression levels after activation of primary HSC by PDGF or TGFβ, normalized to β-actin expression. (**B**) The effect of Epac-1 or PKA activation by respectively 8-pCPT-2'-O-Me-cAMP or 6-Bnz-cAMP on PGDF-induced migration and proliferation in primary HSC. Bars represents mean ± SEM of 3 experiments performed in duplo. **P* < 0.05, ***P* < 0.01.

untreated animals[18]. So, mostly based on *in vitro* studies, it is clear that PGE_2 exerts anti-fibrotic effects via different cells. It is however unclear which cell type is responsible for this effect in the liver. Our study aimed to examine the key effector cells of PGE_2-mediated anti-fibrotic effects and to examine the role of Epac-1 in this cell type. Recent studies indicated an important role of this newly identified cAMP-signaling pathway during fibrogenesis in the lung[23,48].

We found a significant correlation between expression of Epac-1 and ECM formation in the liver and showed the causality of this correlation by administration of PGE_2 to fibrotic mice (fig.1d). To address the question which cell type in the liver is responsible for this Epac-1-mediated effect we applied a cell–specific delivery approach. The multiple effects of PGE_2 on the immune system and in the various hepatic cell types were tackled by us using drug carriers directed at different resident hepatic cells. Local delivery of PGE_2 mimics the physiological situation where PGE_2 is a locally acting mediator.

Three conjugates were synthesized, i.e. PGE_2-LacHSA (PLH), PGE_2-pPBHSA (PPH) and PGE_2-manHSA (PMH) all accumulated *in vivo* in respectively hepatocytes, activated HSC and macrophages. The cell specificity of each of these drug carrier has been extensively reported in the literature[49] and our data show that coupling of PGE_2 to these carriers leads uptake of the construct in the designated cell type. Selective delivery of PGE_2 to the hepatocytes did not affect Epac-1 and collagen deposition. KC-directed PGE_2 also did not affect Epac-1 levels but, induced higher collagen expression levels in contrast to systemically administered native PGE_2 that attenuates collagen deposition. This indicates that the KC is not the effector cell for Epac-1-mediated effects of PGE_2 in advanced liver fibrosis. In macrophages PGE_2 is known to induce a Th2 response which promotes wound healing[42,50], which might be an explanation for the increased collagen levels induced by PGE_2 in KC. However, this effect is independent of Epac-1 activation, since Epac-1 levels did not change in PGE_2-manHSA (PMH)- or man-HSA (MH)-treated animals. Only HSC-selective PGE_2 delivery inhibited the CCl_4-induced scar tissue formation significantly and normalized Epac-1 expression levels. These studies identify the HSC as key effector cell of PGE_2-mediated effects and show the involvement of Epac- 1.

In vitro studies have shown that Epac-1 signaling inhibits Rho-activity, with multiple profibrotic effects, in smooth muscle cells[21,51]. In the liver, Rho-kinase is involved in the regulation and transdifferentiation of HSC via phosphorylation of its downstream mediators, such as myosin light chain (MLC)[35,52]. We found abundant expression of phosphorylated myosin light chain (pMLC) in particular in the fibrotic areas of the liver and only in mice treated with the HSC-selective PGE_2 conjugate, the expression of the pMLC protein was abolished. Our data are in agreement with the general notion that HSC are the key cells in the pathogenesis of liver fibrosis and that inhibition of Rho-kinase activity reduces fibrogenesis[12,52] (Friedman. 2003; Mack et al. 2001; [53]. Our hypothesis that Epac-1 exerts a direct anti-fibrotic effect in HSC was further supported by our *in vitro* assays using primary isolated HSC, showing significant inhibition of PDGF-induced migration and proliferation by the Epac-1 agonist (8-pCPT-2'-O-Me-cAMP). These experiments also confirm the *in vitro* data of Yokoyama *et al*. in HSC[22] that revealed reduced Epac-1 expression levels during activation of HSC. The reduction in Epac-1 levels we found in fibrotic livers (fig 1) is also in agreement with this. Our studies imply an important role of Epac-1 activation in HSC and further delineate the anti-fibrotic effects of the endogenous mediator PGE_2 and its potential mechanism of action *in vivo*.

In summary, our data show that the locally acting mediator PGE_2 exerts different effects in different hepatic cell-types *in vivo*. Only in HSC, PGE_2 treatment induced a reduced ECM deposition *in vivo* which was associated with higher intrahepatic expression levels of the cAMP effector Epac-1 and lower activity of its putative downstream mediator Rho-kinase. These studies support the idea that Epac-1 mediates anti-fibrogenic activities within this cell type. Further studies on the role of the Epac-1 signaling pathway in HSC are needed, but our studies identify this pathway as relevant target for therapies.

ACKNOWLEDGEMENTS

The technical assistance of Mrs. Catharina Reker-Smit is greatly appreciated.

REFERENCES

1. Hirata T, Narumiya S. Prostanoid receptors. *Chem Rev*. 2011;111(10):6209-6230.
2. Dieter P, Altin JG, Decker K, Bygrave FL. Possible involvement of eicosanoids in the zymosan and arachidonic-acid-induced oxygen uptake, glycogenolysis and Ca2+ mobilization in the perfused rat liver. *Eur J Biochem*. 1987;165(2):455-460.
3. Okumura T, Kanemaki T, Kitade H. Stimulation of glucose incorporation into glycogen by E-series prostaglandins in cultured rat hepatocytes. *Biochim Biophys Acta*. 1993;1176(1-2):137-142.
4. Treffkorn L, Scheibe R, Maruyama T, Dieter P. PGE2 exerts its effect on the LPS-induced release of TNF-alpha, ET-1, IL-1alpha, IL-6 and IL-10 via the EP2 and EP4 receptor in rat liver macrophages. *Prostaglandins Other Lipid Mediat*. 2004;74(1-4):113-123.
5. Dieter P, Hempel U, Kamionka S, et al. Prostaglandin E2 affects differently the release of inflammatory mediators from resident macrophages by LPS and muramyl tripeptides. *Mediators Inflamm*. 1999;8(6):295-303.
6. Huang S, Wettlaufer SH, Hogaboam C, Aronoff DM, Peters-Golden M. Prostaglandin E(2) inhibits collagen expression and proliferation in patient-derived normal lung fibroblasts via E prostanoid 2 receptor and cAMP signaling. *Am J Physiol Lung Cell Mol Physiol*. 2007;292(2):L405-13.
7. Hui AY, Cheng AS, Chan HL, et al. Effect of prostaglandin E2 and prostaglandin I2 on PDGF-induced proliferation of LI90, a human hepatic stellate cell

line. *Prostaglandins Leukot Essent Fatty Acids*. 2004;71(5):329-333.

8. Hui AY, Dannenberg AJ, Sung JJ, et al. Prostaglandin E2 inhibits transforming growth factor beta 1-mediated induction of collagen alpha 1(I) in hepatic stellate cells. *J Hepatol*. 2004;41(2):251-258.

9. Heymann F, Trautwein C, Tacke F. Monocytes and macrophages as cellular targets in liver fibrosis. *Inflamm Allergy Drug Targets*. 2009;8(4):307-318.

10. Pinzani M, Rombouts K, Colagrande S. Fibrosis in chronic liver diseases: Diagnosis and management. *J Hepatol*. 2005;42 Suppl(1):S22-36.

11. Friedman SL. Hepatic fibrosis -- overview. *Toxicology*. 2008;254(3):120-129.

12. Friedman SL. Liver fibrosis -- from bench to bedside. *J Hepatol*. 2003;38 Suppl 1:S38-53.

13. Schuppan D, Afdhal NH. Liver cirrhosis. *Lancet*. 2008;371(9615):838-851.

14. Mallat A, Gallois C, Tao J, et al. Platelet-derived growth factor-BB and thrombin generate positive and negative signals for human hepatic stellate cell proliferation. role of a prostaglandin/cyclic AMP pathway and cross-talk with endothelin receptors. *J Biol Chem*. 1998;273(42):27300-27305.

15. Swaney JS, Roth DM, Olson ER, Naugle JE, Meszaros JG, Insel PA. Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. *Proc Natl Acad Sci U S A*. 2005;102(2):437-442.

16. Weinberg E, Zeldich E, Weinreb MM, Moses O, Nemcovsky C, Weinreb M. Prostaglandin E2 inhibits the proliferation of human gingival fibroblasts via the EP2 receptor and epac. *J Cell Biochem*. 2009;108(1):207-215.

17. Haag S, Warnken M, Juergens UR, Racke K. Role of Epac1 in mediating anti-proliferative effects of prostanoid EP(2) receptors and cAMP in human lung fibroblasts. *Naunyn Schmiedebergs Arch Pharmacol*. 2008;378(6):617-630.

18. Ruwart MJ, Wilkinson KF, Rush BD, et al. The integrated value of serum procollagen III peptide over time predicts hepatic hydroxyproline content and stainable collagen in a model of dietary cirrhosis in the rat. *Hepatology*. 1989;10(5):801-806.

19. de Rooij J, Zwartkruis FJ, Verheijen MH, et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*. 1998;396(6710):474-477.

20. Grandoch M, Roscioni SS, Schmidt M. The role of epac proteins, novel cAMP mediators, in the regulation of immune, lung and neuronal function. *Br J Pharmacol*. 2010;159(2):265-284.

21. Roscioni SS, Elzinga CR, Schmidt M. Epac: Effectors and biological functions. *Naunyn Schmiedebergs Arch Pharmacol*. 2008;377(4-6):345-357.

22. Yokoyama U, Patel HH, Lai NC, Aroonsakool N, Roth DM, Insel PA. The cyclic AMP effector epac integrates pro- and anti-fibrotic signals. *Proc Natl Acad Sci U S A*. 2008;105(17):6386-6391.

23. Huang SK, Wettlaufer SH, Chung J, Peters-Golden M. Prostaglandin E2 inhibits specific lung fibroblast functions via selective actions of PKA and epac-1. *Am J Respir Cell Mol Biol*. 2008;39(4):482-489.

24. Kojima F, Kapoor M, Kawai S, Yang L, Aronoff DM, Crofford LJ. Prostaglandin E2 activates Rap1 via EP2/EP4 receptors and cAMP-signaling in rheumatoid synovial fibroblasts: Involvement of Epac1 and PKA. *Prostaglandins Other Lipid Mediat*. 2009;89(1-2):26-33.

25. Beljaars L, Poelstra K, Molema G, Meijer DK. Targeting of sugar- and charge-modified albumins to fibrotic rat livers: The accessibility of hepatic cells after chronic bile duct ligation. *J Hepatol*. 1998;29(4):579-588.

26. Beljaars L, Weert B, Geerts A, Meijer DK, Poelstra K. The preferential homing of a platelet derived growth factor receptor-recognizing macromolecule to fibroblast-like cells in fibrotic tissue. *Biochem Pharmacol*. 2003;66(7):1307-1317.

27. Poelstra K, Schuppan D. Targeted therapy of liver fibrosis/cirrhosis and its complications. *J Hepatol*. 2011;55(3):726-728.

28. Jansen RW, Molema G, Ching TL, et al. Hepatic endocytosis of various types of mannose-terminated albumins. what is important, sugar recognition, net charge, or the combination of these features. *J Biol Chem*. 1991;266(5):3343-3348.

29. Melgert BN, Olinga P, Van Der Laan JM, et al. Targeting dexamethasone to kupffer cells: Effects on liver inflammation and fibrosis in rats. *Hepatology*. 2001;34(4 Pt 1):719-728.

30. Borkham-Kamphorst E, Kovalenko E, van Roeyen CR, et al. Platelet-derived growth factor isoform expression in carbon tetrachloride-induced chronic liver injury. *Lab Invest*. 2008;88(10):1090-1100.

31. Wong L, Yamasaki G, Johnson RJ, Friedman SL. Induction of beta-platelet-derived growth factor receptor in rat hepatic lipocytes during cellular activation in vivo and in culture. *J Clin Invest*. 1994;94(4):1563-1569.

32. Hagens WI, Mattos A, Greupink R, et al. Targeting 15d-prostaglandin J2 to hepatic stellate cells: Two options evaluated. *Pharm Res*. 2007;24(3):566-574.

33. Yee HF,Jr. Rho directs activation-associated changes in rat hepatic stellate cell morphology via regulation of the actin cytoskeleton. *Hepatology*. 1998;28(3):843-850.

34. Charest PG, Firtel RA. Big roles for small GTPases in the control of directed cell movement. *Biochem J*. 2007;401(2):377-390.

35. Pellegrin S, Mellor H. Actin stress fibres. *J Cell Sci*. 2007;120(Pt 20):3491-3499.

36. Klein S, Van Beuge MM, Granzow M, et al. HSC-specific inhibition of rho-kinase reduces portal pressure in cirrhotic rats without major systemic effects. *J Hepatol*. 2012;57(6):1220-1227.

37. Roscioni SS, Kistemaker LE, Menzen MH, et al. PKA and epac cooperate to augment bradykinin-induced interleukin-8 release from human airway smooth muscle cells. *Respir Res*. 2009;10:88.

38. Roscioni SS, Maarsingh H, Elzinga CR, et al. Epac as a novel effector of airway smooth muscle relaxation. *J Cell Mol Med*. 2011;15(7):1551-1563.

39. Sheibanie AF, Khayrullina T, Safadi FF, Ganea D. Prostaglandin E2 exacerbates collagen-induced arthritis in mice through the inflammatory interleukin-23/interleukin-17 axis. *Arthritis Rheum*. 2007;56(8):2608-2619.

40. Yao C, Sakata D, Esaki Y, et al. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat Med*. 2009;15(6):633-640.

41. Sheibanie AF, Tadmori I, Jing H, Vassiliou E, Ganea D. Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. *FASEB J*. 2004;18(11):1318-1320.

42. Chen F, Liu Z, Wu W, et al. An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. *Nat Med*. 2012;18(2):260-266.

43. Nataraj C, Thomas DW, Tilley SL, et al. Receptors for prostaglandin E(2) that regulate cellular immune responses in the mouse. *J Clin Invest*. 2001;108(8):1229-1235.

44. Tajima T, Murata T, Aritake K, et al. EP2 and EP4 receptors on muscularis resident macrophages mediate LPS-induced intestinal dysmotility via iNOS upregulation through cAMP/ERK signals. *Am J Physiol Gastrointest Liver Physiol*. 2012;302(5):G524-34.

45. Ratcliffe MJ, Walding A, Shelton PA, Flaherty A, Dougall IG. Activation of E-prostanoid4 and E-prostanoid2 receptors inhibits TNF-alpha release from human alveolar macrophages. *Eur Respir J*. 2007;29(5):986-994.

46. Refsnes M, Thoresen GH, Dajani OF, Christoffersen T. Stimulation of hepatocyte DNA synthesis by prostaglandin E2 and prostaglandin F2 alpha: Additivity with the effect of norepinephrine, and synergism with epidermal growth factor. *J Cell Physiol*. 1994;159(1):35-40.

47. Kimura M, Osumi S, Ogihara M. Prostaglandin E(2) (EP(1)) receptor agonist-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes: The involvement of TGF-alpha. *Endocrinology*. 2001;142(10):4428-4440.

48. Roscioni SS, Dekkers BG, Prins AG, et al. cAMP inhibits modulation of airway smooth muscle phenotype via the exchange protein activated by cAMP (epac) and protein kinase A. *Br J Pharmacol*. 2011;162(1):193-209.

49. Poelstra K, Prakash J, Beljaars L. Drug targeting to the diseased liver. *J Control Release*. 2012;161(2):188-197.

50. Liu L, Ge D, Ma L, et al. Interleukin-17 and prostaglandin E2 are involved in formation of an M2 macrophage-dominant microenvironment in lung cancer. *J Thorac Oncol*. 2012;7(7):1091-1100.

51. Zieba BJ, Artamonov MV, Jin L, et al. The cAMP-responsive Rap1 guanine nucleotide exchange factor, epac, induces smooth muscle relaxation by down-regulation of RhoA activity. *J Biol Chem*. 2011;286(19):16681-16692.

52. Mack CP, Somlyo AV, Hautmann M, Somlyo AP, Owens GK. Smooth muscle differentiation marker

gene expression is regulated by RhoA-mediated actin polymerization. *J Biol Chem*. 2001;276(1):341-347.

53. Fukushima M, Nakamuta M, Kohjima M, et al. Fasudil hydrochloride hydrate, a rho-kinase (ROCK) inhibitor, suppresses collagen production and enhances collagenase activity in hepatic stellate cells. *Liver Int*. 2005;25(4):829-838.

CHAPTER 6

Intestinal Alkaline Phosphatase activity affects intrahepatic macrophage activation and fibrogenesis.

Marlies Schippers[1], Eduard Post[1], Aysegul Cetintas[1], Catharina Reker-Smit[1], Madhu S. Malo[2], Richard Hodin[2], José Luis Millán[3], Leonie Beljaars[1] Klaas Poelstra[1].

[1] Department of Pharmacokinetics, Toxicology and Targeting, University of Groningen, The Netherlands
[2] Department of Surgery, Massachusetts General Hospital, Harvard MedicalSchool, Boston, MA 02114
[3] Sanford-Burnham Medical Research Institute, Sanford Children's Health Research Center, La Jolla, CA 92037

ABSTRACT

Serum alkaline phosphatase (AP) levels serve as markers for many liver diseases. Some studies indicate that AP may act as a protective enzyme by dephosphorylation of LPS thereby detoxifying it. Gut-derived LPS is known to aggravate liver damage and fibrosis and we hypothesized that increased AP levels may represent a physiological response to LPS. We now studied the role of intestinal-AP and LPS in the liver using an intestinal-AP (iAP) KO model and also examined effects of exogenous iAP administration in mice after acute and chronic CCl_4-induced fibrosis. In iAP KO mice, higher wound healing activity was found as compared to WT mice. This observation was paralleled by an increase in macrophages of the pro-fibrotic M2 phenotype. Inhibition of LPS production by antibiotics abolished these changes. In fibrotic human livers and in mice with CCl_4-induced acute and chronic liver damage, high AP activity was found along hepatocytes and fibrotic bands. This AP-activity was able to dephosphorylate the lipid A moiety of LPS. We further explored the role of AP by a 2-week iAP treatment in mice with CCl_4-induced fibrosis. In iAP-treated mice, significantly lower desmin positive cells were found associated with attenuated accumulation of M2 macrophages as compared to controls. During acute liver damage, however, iAP administration was associated with enhanced desmin staining and M2 accumulation, indicating wound repair. **Conclusions**. This study provides evidence that AP acts as an important, protective enzyme that regulates acute and chronic fibrogenic processes in the liver in different ways. In the acute phase, AP induces wound healing to repair the damaged areas whereas it attenuates collagen deposition in the chronically diseased fibrotic liver. Our data also indicate that these regulatory effects are due to detoxification of LPS.

INTRODUCTION

The liver is the first-pass organ for gut derived toxins and foreign particles. It is becoming increasingly clear that these gut-derived products affect liver fibrogenesis, partly because the permeability of the intestinal wall changes during fibrogenesis[1,2]. In a healthy situation, small amounts of gut-derived lipopolysaccharide (LPS) are taken up by hepatocytes and Kupffer cells without significant inflammation in the liver. However, hepatocyte damage and activation of resident hepatic macrophages during fibrogenesis, combined with an increased uptake of bacterial products, may further aggravate liver damage and fibrosis[1,3].

Liver fibrosis is characterized by a disarranged liver architecture as a result of abundant scar tissue formation produced by activated hepatic stellate cells (HSC). It is becoming clear that macrophages play an important role in the regulation of inflammation and activation of HSC[4-7]. Macrophages regulate these processes by polarization into the pro-inflammatory, classically activated macrophage (M1) phenotype or the alternative activated macrophage (M2) phenotype[4,8], with anti-inflammatory activities. LPS is known to initiate M1 polarization and induce a Th1 response, which is counterbalanced by accumulation of M2 macrophages with wound healing capacities. Persistent exposure of LPS to the liver may result in changes of M1/M2 polarization and subsequently, chronic inflammation and fibrogenesis. LPS may also directly activate HSC, further promoting fibrogenesis[9].

Liver fibrosis is associated with high levels of serum alkaline phosphatase (AP). Serum AP levels are often monitored as biochemical marker of a number of pathologies, most often bone and liver diseases. Of the four AP isozymes that exist in humans, tissue-nonspecific AP (TNAP, a.k.a liver, bone, kidney AP), intestinal AP (iAP), placental AP (PLAP) and germ cell AP (GCAP), serum AP levels in a normal individual are comprised of a mixture of the bone and liver isoforms of TNAP, and a small variable contribution from the iAP and GCAP isozymes[10].

Interestingly, in mice, serum AP activity is mainly comprised of bone-derived TNAP isoform with smaller contributions from two intestinal isozymes, encoded by the Akp3 and Akp6 genes, as normal mouse hepatocytes are devoid of TNAP activity[11]. An increase in serum AP, and in particular an increase in the percentage of liver-derived TNAP isoform is used as a marker for acute liver damage as well as chronic liver diseases. The physiological role of this increase in AP levels is however completely unknown.

Recent studies indicate that both TNAP and iAP may act as a protective enzymes by dephosphorylation of the lipid A moiety of LPS resulting in a non-toxic molecule[12-15]. We hypothesized that high levels of AP found during liver fibrosis or acute liver damage may represent a physiological response to higher levels of LPS. In this study we therefore investigated the role of intestinal AP (iAP) on liver fibrogenesis using iAP knock out mice (iAP KO; *Akp3*$^{-/-}$ mice)[16] and subsequently studied HSC activation, fibrogenesis and M1/M2 composition. The antibiotics neomycin and Polymyxin B, both affecting gram-negative bacteria were used to reduce intestinal LPS levels in the iAP KO animals. In addition, we examined macrophage activity and fibrogenesis in fibrotic mice after administration of iAP to mice with CCl_4 induced acute and chronic liver damage. Our study shows that intestinal and hepatic AP affect hepatic macrophage activity and that AP plays an important role as a protective enzyme in the gut-liver axis.

MATERIALS & METHODS

The following primary antibodies were used: goat anti-collagen I (Southern Biotechnology Associates, Birmingham, AL), goat anti-Desmin (Santa Cruz Biotecnology, Heidelberg, Germany), goat anti-chitinase 3-like/ECF-L (YM-1), rabbit anti-IRF5 (Protein Tech, Manchester, UK), mouse anti-PAR (BD Biosciences, Breda, Netherlands), Species-specific horseradish peroxidase-conjugated secondary antibodies were purchased from Dako Denmark A/S (Glostrup, Denmark).

Animal Experiments

All animal experiments were approved by the Animal Ethics Committee of the University of Groningen, the Netherlands. Animals were purchased from Harlan (Zeist, Netherlands).

IAP-KO mice (*Akp3*-/-, *Mus musculus* C57BL/6)[17] were obtained from the Sanford-Burnham Medical Research Institute (La Jolla, CA) and bred at the Massachusetts General Hospital (MGH, Boston, MA) animal facility to create homozygous IAP-KO, heterozygous, and wild-type C57BL/6 (WT) littermates.

Genotype was confirmed by polymerase chain reaction analysis. Animals were maintained in a specific pathogen-free environment at MGH in accordance with the guidelines of the Committee on Animals of Harvard Medical School (Boston, MA). All experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) and according to regulations of the Subcommittee on Research Animal Care of the MGH and the National Institutes of Health (publication no. 85-23,167 NIH, 1985).

Intestinal-alkaline Phosphatase knock out model (iAP KO): Male Akp3$^{-/-}$ mice received saline or Neomycin (220 mg/kg/day) plus PolymyxinnB (100 mg/kg/day) in their drinking water from week 4 to week 6 and were sacrificed at the age of 6 weeks (n=6/group). Age- and sex-matched untreated wild type (WT) C57BL/6 mice served as control (n=6/group).

CCl_4-induced acute liver fibrosis model: Male Balb/c mice (20-22 g) received a single intraperitoneal injection of CCl_4 diluted in olive oil (0.5 ml/kg). After 3h and 13h mice were treated with Calf-Intestinal Alkaline Phosphatase (kindly provided by AM-Pharma BV, the Netherlands) (500 mUnits/mouse, i.v.) or saline vehicle (n=6-7 per group). Mice were sacrificed 24h after the CCl_4 injection.

CCl_4-induced advanced liver fibrosis model: Male Balb/c mice (20-22 g) were injected twice a week intraperitoneally with saline or increasing doses of CCl_4 diluted in olive oil (week 1, 0.5 mL/kg; week 2, 0.8 mL/kg and week 3-8, 1 mL/kg prepared in olive oil). At week 7 and 8, mice were treated intravenously with Calf-Intestinal Alkaline Phosphatase (500 mUnits /mouse, thrice per week) or saline vehicle, n = 6-9 per group. All mice were sacrificed at week 8. AP and ASAT levels were measured by standard automated laboratory methods.

Immunohistochemistry and quantitative analysis of sections: Immunohistochemistry was performed on 4 µm cryostat sections according to standard indirect immunoperoxidase methods. Stainings were visualized using 3-amino-9-ethylcarbazole or NovaRed (Vector Laboratories). Nuclei were counter-

stained with Mayer's hematoxylin. Immunohistochemical stainings were quantified by analysing complete sections from 3 different liver lobes of each animal at magnification 10x10 using the Cell D image analysing software (Olympus, Hamburg, Germany).

Western Blot Analysis: Liver homogenates were subjected to SDS-PAGE (12%) and the separated proteins were transferred to PVDF membrane. The membranes were blocked with TBST (20 mM TrisHCl, pH7.6, 154 mM NaCl, 0.1% Tween20) containing 5% skimmed milk and incubated with primary antibody. After washings, the blots were incubated with HRP-conjugated secondary anti-bodies (DAKO, Glostrup, Denmark). Finally, the blots were developed using Western Lightning-ECL reagent (Perkin-Elmer, Boston, MA) according to the manufacturer's instructions. Blots were quantified with Genetools (Syngene, Cambridge, UK). Samples from 3 different liver lobes of each animal were analyzed.

Alkaline Phosphatase staining: 0.1% Fast Blue and 0.02% Naphtol AS-MX were dissolved in 0.1 M TRIS/HCl pH 8.2 buffer containing 2 mM $MgCl_2$. The solution was filtrated and incubated on cryo-sections for 30 minutes at 37 °C. Sections were washed in PBS and nuclei were counterstained with Mayer's hematoxylin. Control sections were stained with levamisole, which is an inhibitor of liver/bone/kidney AP[12] to exclude the detection of intestinal AP administered to some groups. The latter iso-enzyme is not inhibitable by levamisole[12].

Histochemical detection of LPS: LPS-dephosphorylating activity of endogenous AP in rat livers was examined by incubating cryostat sections with LPS (from *Escherichia coli* (serotype O55:B5), Sigma (St. Louis, MO), as a substrate. Briefly, sections were fixed in 4% formalin-macrodex and subsequently incubated for 120 min in Tris-HCl buffer (pH 7.6) containing LPS (final concentration: 3.2 mg/ml), $MgSO_4$ (final concentration: 0.01 M) and $Pb(NO_3)_2$ [final concentration: 0.06% (wt/vol)] at 37°C. A lead phosphate precipitate is formed at the site of enzyme activity, which is converted by incubation with Na_2S to a lead sulphate precipitate, which appears as a dark brown staining. Specificity of this staining has previously been demonstrated by inhibition of AP activity using levamisole[12]. LPS was omitted in control incubations.

Quantitative analysis Alkaline Phosphatase: Alkaline phosphatase was measured in livers of mice using QUANTI-Blue reagent (InvivoGen, Dan Diego, USA) according to the manufacture''s instructions.

Statistical analysis

Results are expressed as the mean ± SD, unless otherwise specified. Statistical analyses were performed using the Mann-Whitney *t* test. $p < 0.05$ was considered as the minimum level of significance.

RESULTS

Lack of intestinal AP induces liver fibrogenesis

Intestinal AP knock out (iAP KO; *Akp3*$^{-/-}$ mice) mice were used to examine the role of iAP in liver physiology. In 6-week-old iAP KO mice, mild but significant changes in fibrogenic markers and macrophage activity were found compared to wild type (WT) mice. Higher expression levels of the early fibrogenic marker Protease-Activated Receptor-1 (PAR-1) as well as collagen-1 were found in

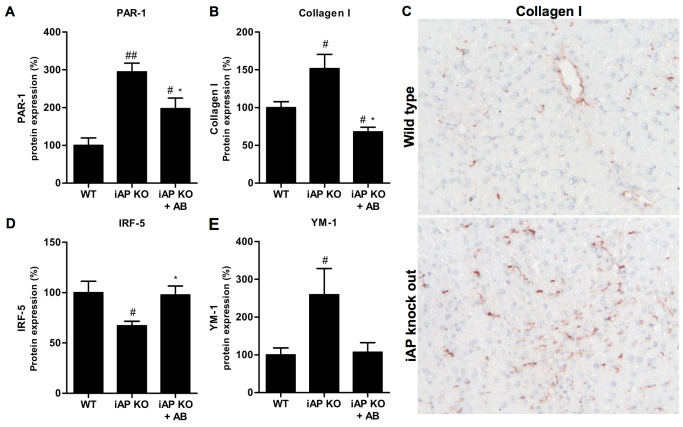

Figure 1. Intrahepatic fibrosis and macrophage markers in iAP KO and wild-type mice. (**A**) Intrahepatic expression levels of the early fibrotic marker PAR-1 and (**B**) ECM marker collagen-1 after quantitative analysis of western blots. (**C**) Representative pictures of the staining for collagen 1. Western blot analysis of (**D**) M1 and (**E**) M2 macrophage accumulation in normal (WT) livers and livers of iAP KO mice with and without antibiotics (AB) Neomycin plus Polymyxin B. IRF-5 staining was used as marker for M1 macrophages [15], YM-1 as marker for M2 macrophages[17,40]. Bars represent mean ± SEM of 6 mice per group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus WT mice. $^{*}P < 0.05$, versus iAP KO mice.

livers of iAP KO mice compared to WT mice, as analyzed by western blot (fig.1a,b). Immunohistochemical staining confirmed this increased hepatic collagen deposition in iAP KO mice (fig.1c).

We hypothesized that the increment of these parameters was induced by gut-derived LPS in mice with a disrupted iAP gene. To study this, we treated animals orally with the antibiotics Neomycin (N) and Polymyxin B (P). Neomycin selectively kills gram-negative bacteria, whereas Polymyxin B binds LPS. Absorption of both antibiotics from the intestine is absent to very poor (<4%) thus excluding any direct effects of these drugs on the liver. Western blot analysis showed significantly lower expression levels of PAR-1 and collagen-1 in iAP KO mice treated with a combination of N and P compared to untreated iAP KO mice (fig.1a,b).

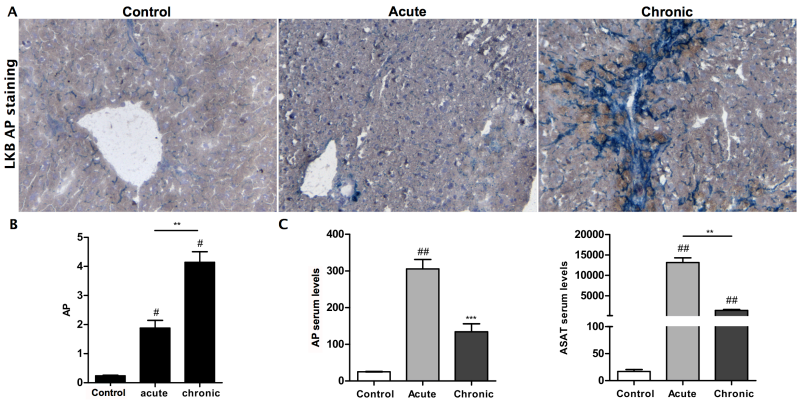

Figure 2. Intrahepatic and serum AP- activity after acute damage and chronic fibrogenesis in CCl_4-treated mice. (**A**) Representative pictures of AP- activity in normal mice and mice with acute liver damage or fibrosis. (**B**) Quantitative analysis of intrahepatic AP activity and (**C**) serum levels in normal mice, after acute liver damage and liver fibrosis. Bars represent mean ± SEM of 6-7 mice per group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus normal control mice. $^{**}P < 0.01$, $^{***}P < 0.001$versus CCl_4-treated mice.

Subsequently, we investigated macrophage polarization in iAP KO mice, with and without antibiotics and compared the data with WT mice. We used interferon-regulatory factor-5 (IRF-5) to identify the M1 macrophages[18]. IRF-5 is involved in the activation of genes encoding inflammatory cytokines such as INFγ, TNFα, IL-6, IL12 and IL23[18]. Chitinase-like secretory protein (YM-1) was used to detect M2 macrophages. YM-1 is significantly upregulated after macrophage activation by IL4 and/or IL13[19-21]. Lower expression levels of IRF-5+ M1 macrophages were observed in livers of iAP KO mice compared to WT (fig.1d) whereas the accumulation of YM-1 positive M2 macrophages was up-regulated in these livers compared to WT (fig.1e). Treatment of iAP KO mice with a combination of N and P abolished this effect; expression levels for both the M1 maker and the M2 marker completely returned to normal values compared to untreated iAP mice.

It appears that the lack of iAP stimulated wound repair mechanisms within the liver which was paralleled by a shift in M1/M2 balance towards the M2 phenotype. Treatment of iAP KO mice with oral antibiotics normalized wound repair and macrophage polarization.

Intrahepatic AP expression is gradually increased during fibrogenesis

Intrahepatic AP expression levels were subsequently examined in WT mice, after CCl_4-induced acute liver damage and after induction of chronic CCl_4-induced fibrogenesis. Intrahepatic AP activity was increased after acute damage and was further increased in mice with CCl_4-induced fibrosis (fig. 2a,b). Immunohistochemical staining showed that AP was localized around blood vessels and bile canaliculi in normal mice as previously reported[11] . In livers with acute and chronic injury, additional AP expression was found in the necrotic areas respectively along the fibrotic bands (fig.2a). Quantification of hepatic AP expression confirmed the immunohistochemistry results (fig.2b). Sub-

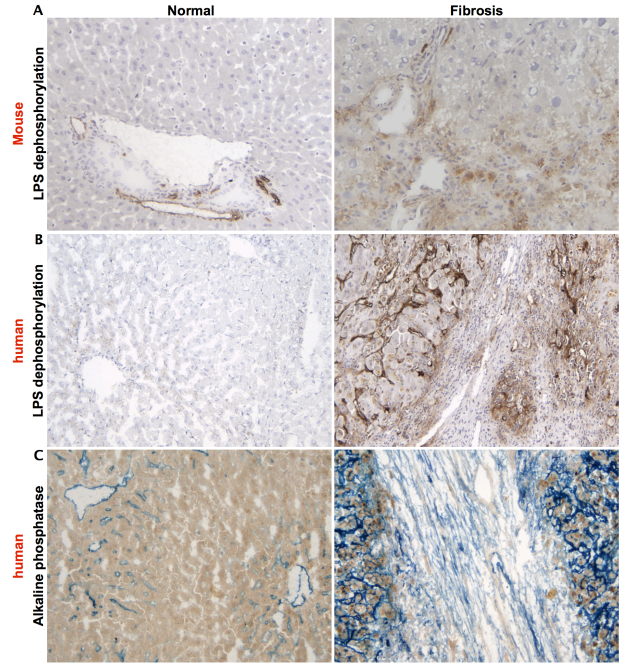

Figure 3. LPS-dephosphorylation and AP-activity in normal and fibrotic livers of mouse and man. Representative pictures of LPS dephosphorylating activity in livers of (**A**) mice and (**B**) man. Figure **C** shows AP-activity in human livers. Note that the LPS-dephosphorylating activity is co-localized with the AP-activity. This activity was mainly found along the plasma membranes of hepatocytes and in the fibrotic areas.

sequently, we measured serum levels of AP and ASAT, the latter as a marker for liver damage. Serum levels of AP as well as ASAT were significantly increased in mice with diseased livers as compared to serum levels of normal mice. AP and ASAT serum levels were highest in mice with acute liver damage (fig.2c).

AP-activity is able to dephosphorylate the lipid A moiety of LPS in livers of mouse and man

The phosphate groups of the lipid A molecule greatly determine the toxicity of LPS[22-25]. We[13-15,26] and others[12,27,28] have found that AP is able to remove these phosphate groups from lipid A resulting in its detoxification. We now investigated whether this intrahepatic AP-activity is able to dephosphorylate the lipid A moiety of LPS in the fibrotic liver. In normal mice, histochemical staining showed clear phosphate release around the bile ducts and arteries, the sites of TNAP expression in the normal mouse liver, after incubation with diphosphoryl lipid A, which represents a chemically well defined toxic part of LPS. In fibrotic livers this phosphate-releasing activity was greatly enhanced, particularly along the fibrotic bands where strong LPS-dephosphorylating activity was found (fig.3a).

We also examined human fibrotic livers and found increased LPS-dephosphorylating activity compared to normal human livers. This activity was mainly localized along the plasma membranes of hepatocytes and in the fibrotic areas (fig.3b). Subsequently we stained AP-activity in normal and fibrotic human livers and again found higher levels of AP-activity in fibrotic compared to normal livers. Localization of AP activity in these livers corresponded with the LPS-dephosphorylating activity. (fig.3c). In summary, high levels of AP-activity and LPS dephosphorylating activity were found in fibrotic livers of mice and man, and both these enzyme activities were primarily localized along bile ducts, hepatocytes and in the fibrotic areas.

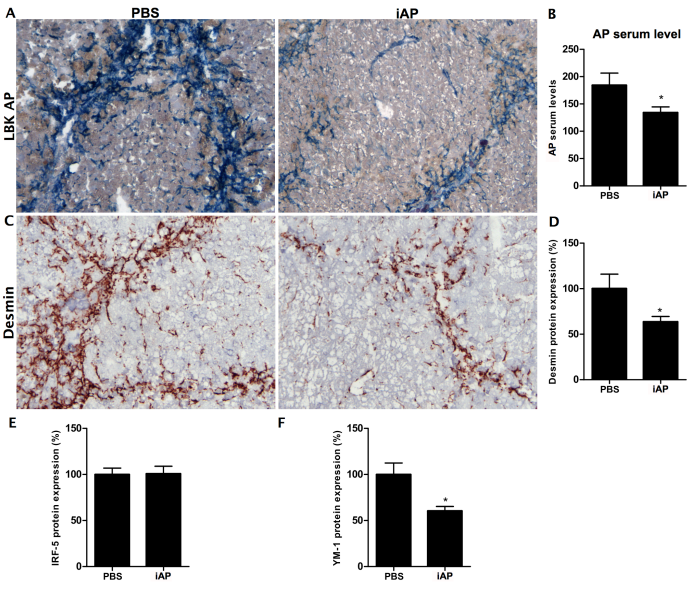

Figure 4; Effect of exogenous iAP on CCl_4-induced liver fibrosis. (**A**) Representative pictures (magnification 100x) of intrahepatic AP-activity and (**B**) AP serum levels in mice after 8 weeks of administration of CCl_4. Animals received either vehicle (control) or iAP from week 6 to week 8. (**C**) Representative pictures and (**D**) quantification of hepatic desmin staining. (**E**) Intrahepatic IRF-5 and (**F**) YM-1 expression levels after iAP or vehicle treatment in the chronic phase of fibrogenesis as analyzed by western blot. Bars represent mean ± SEM of 6-7 mice per group. *$P < 0.05$ versus CCl_4-treated mice.

Fibrogenesis is reduced in mice after iAP administration

We further explored the role of AP by injecting iAP to mice with CCl_4-induced liver fibrosis. Intestinal AP is taken up by the asioalglycoprotein receptor in hepatocytes and has a half-life of several minutes in serum[26]. Mouse livers were examined at a time point when exogenously administered iAP had been cleared[29].

24 hr after injection of iAP, immunohistochemical staining showed less hepatic AP activity (fig.4a) in iAP-treated fibrotic mice compared to untreated fibrotic mice In addition, a reduction in serum AP levels was observed in iAP-treated mice compared to untreated fibrotic mice (fig.4b). The staining for AP within the livers was completely inhibitable by the AP-inhibitor levamisole, confirming that this activity was derived from hepatic AP, and not from the administered intestinal AP which is resistant to levamisole[14]. This reduction in hepatic AP-activity after treatment with iAP was paralleled by significantly lower expression levels of desmin-positive cells (fig.4c,d). Analysis of the macrophage profile revealed a significantly lower accumulation of YM-1+ M2 macrophages in iAP-treated mice compared to untreated fibrotic mice (fig.4f). No changes were observed in IRF-5+ cells in livers of treated and untreated mice (fig.4e). So, during chronic injury an increased influx of M1 and M2 macrophages was found in CCl_4 administered mice compared to control mice and iAP-treatment reduced M2 accumulation, associated with a significant reduction in the number of desmin-positive fibroblasts.

iAP promotes repair of damaged tissue after acute liver damage

If the increment of AP is a physiological, protective response of the liver upon damage, then administration of iAP to mice with acute liver damage may also be beneficial. Indeed, immunohistochemical staining showed higher levels of desmin-positive cells reflecting HSC activation and repair of the damaged areas after iAP administration (fig.5c,d). This was associated with a higher accumulation of M2 macrophages (fig.5f), whereas M1 expression levels remained unchanged (fig.5e). No significant changes in intrahepatic AP expression or serum levels were observed (fig.5a,b). It ap-

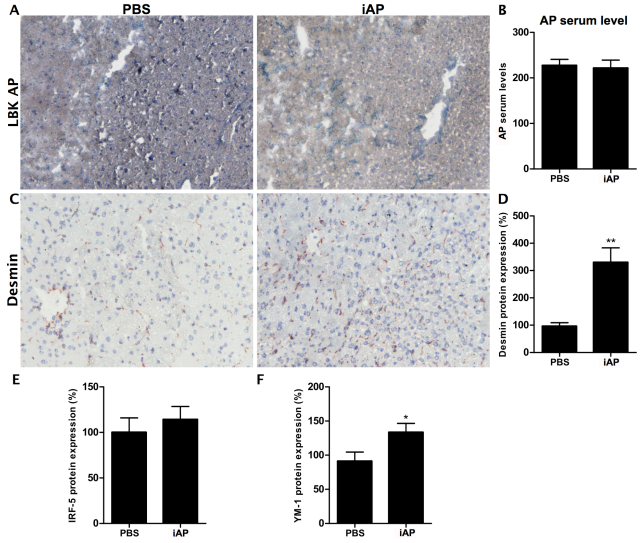

Figure 5; Effect of exogenous iAP on acute liver damage. (**A**) Representative pictures (magnification 100x) of intrahepatic AP-activity and (**B**) AP serum levels, 24 hr after CCl_4 administration in animals receiving either vehicle (control) or iAP, 3 and 13 hr after the CCl_4 injection. (**C**) Representative pictures and (**D**) quantification of hepatic desmin staining. (**E**) Intrahepatic IRF-5 and (**F**) YM-1 expression levels after iAP or vehicle treatment in mice with acute liver damage as analyzed by western blot. Bars represent mean ± SEM of 6-7 mice per group. *$*P$* < 0.05, *$**P$* < 0.01 versus CCl_4-treated mice.

pears, therefore, that AP administration to mice with acute liver damage enhanced wound healing activity, necessary to repair the damaged areas, combined with a higher accumulation of M2 macrophages compared to CCl_4-treated animals receiving no iAP.

Discussion

The present data indicate that iAP significantly affects macrophage- and wound repair activities within the liver in mice. iAP is a small intestinal brush border enzyme and several studies have shown that this enzyme is able to detoxify LPS derived from Gram-negative bacteria[12-14,26,28]. This gut mucosal defence factor is influenced by enteral nutrition[27].

Exogenous iAP has been shown to reduce LPS-mediated toxicity[28,30], restores gut microbiota and reduces pathogen colonisation[31]. The mechanism of action of AP in this context is the dephosphorylation of the lipid A moiety of LPS, which represents the toxic part of the molecule and is well preserved among many gram-negative bacterial strains. Our data support and extend previous data, further defining AP as a protective enzyme with regulatory effects on biological processes and as an important inducer of homeostasis.

During chronic liver diseases, gut-derived LPS is known to promote the fibrogenic process due to increased permeability of the intestinal wall associated with increased levels of permeability enhancing factors in the circulation[32]. In a NASH animal model, externally-induced colitis led to higher portal LPS levels which were followed by hepatic inflammation and fibrogenesis[33]. Due to its ability to detoxify LPS, we hypothesized that iAP may protect the liver against LPS-mediated effects. Indeed, livers of iAP KO mice contained higher levels of collagen I and PAR-1 as compared to the WT mice. Reduction of intestinal LPS levels using a combination of the non-absorbable antibiotics (Neomycin and Polymyxin B) resulted in normalisation of collagen I and PAR-1 levels in iAP KO mice, implicating a role for gut-derived LPS in this process. These data provide additional evidence for the importance of gut-derived mediators, as well as the role for iAP in the pathogenesis of liver fibrosis.

These data raised the question whether liver AP, which is routinely measured as a marker for liver damage or chronic liver diseases such as cholestasis, cirrhosis, hepatitis, fatty liver disease, cholangitis and chronic diseases[34] may act as a protective enzyme in the liver by dephosphorylation of LPS. In this study we found significantly higher activity for hepatic AP during fibrogenesis in mice and man compared to normal livers. AP-activity was most prominent along hepatocytes and fibrotic bands and was co-localized with LPS-dephosphorylating activity in both species.

Given that under normal physiological conditions hepatocytes express either no (in the mouse) or low (in humans) levels of TNAP, and that LPS administration enhances the AP activity in hepatocytes[26], suggests that increased serum AP levels during liver diseases are not simply a consequence of passive release from damaged liver cells, but reflects *de novo* synthesis and local expression in the diseased areas. In the present study, serum AP levels do not parallel intrahepatic AP expression levels (figure 2), consistent with our hypothesis that the increased AP expression during acute and chronic liver disease is a physiological response of the liver to LPS.

Because LPS is an important inducer of macrophage responses we studied macrophage polarization into their M1 and M2 phenotypes *in vivo*. M1 macrophages are known to have microbicidal or tumoricidal capacities, are activated by IFNγ and TNFα or LPS[8,18,35] and they release metalloproteinases (MMPs) that degrade scar tissue[36,37]. This process is counterbalanced by M2 macrophages which have immunosuppressive actions, induce wound healing by fibroblast activation and secrete tissue remodelling enzymes including tissue inhibitors of metalloproteinase (TIMPs) and precursors for ECM proteins[4,8,35,37-39]. We found that in iAP KO mice the intrahepatic M1/M2 balance was shifted towards the wound healing M2 macrophages, which is in line with enhanced liver fibrogenesis observed in these mice. In general, higher accumulation of M1 macrophages are expected as a response to increased levels of LPS[35,40]. However, it is well known that a Th1 (acute inflammation) response is followed by a Th2 (repair) response[8,41]. In 6 week old iAP KO mice, the LPS-triggered inflammatory state is apparently followed by such a Th2 repair response. In time, collagen deposition may increase although no reports indicate that this KO-model is associated with a fibrotic liver. It is likely that more triggers are needed in order to reach such a state.

The increased hepatic AP expression during acute damage and fibrosis, and the protective effects of gut-derived AP against inflammation and wound healing suggest an important regulatory role of AP during fibrogenesis. We tested this hypothesis by administration of iAP to mice with acute liver damage and CCl_4-induced fibrosis and found that AP exerts protective actions in both cases and steers the process towards homeostasis. In the acute phase, exogenous AP stimulates wound healing combined with a high M2 accumulation. In this phase AP may inhibit inflammation by the detoxification of LPS and stimulate repair of the necrotic areas. This finding is supported by the fact that AP is found to inhibit the pro-inflammatory nucleotide uridine diphosphate in mice[16]. Increased permeability of the intestinal wall and a higher uptake of LPS aggravate liver damage and fibrosis[1,2,42]. During chronic fibrogenesis, higher levels of hepatic AP were found in the fibrotic bands and administration of iAP resulted in a reduced collagen deposition associated with lower accumulation of M2.

In summary, this study provides evidence that AP acts as an important, protective enzyme that regulates acute and chronic fibrogenic processes in the liver in different ways. In the acute phase, AP induces wound healing processes to repair the damaged areas whereas it attenuates collagen deposition in the chronically diseased fibrotic liver. These regulatory effects may be due to detoxification of LPS. In the acute phase as well as in the chronic phase, AP inhibits LPS-induced M1 macrophage triggering. Our study shows that iAP activity dramatically affects liver physiology, indicating that this enzyme plays an important protective role in the gut-liver axis.

REFERENCES

1. Enomoto N, Ikejima K, Yamashina S, et al. Kupffer cell sensitization by alcohol involves increased permeability to gut-derived endotoxin. *Alcohol Clin Exp Res*. 2001;25(6 Suppl):51S-4S.
2. Enomoto N, Ikejima K, Bradford BU, et al. Role of kupffer cells and gut-derived endotoxins in alcoholic liver injury. *J Gastroenterol Hepatol*. 2000;15 Suppl:D20-5.
3. Pradere JP, Troeger JS, Dapito DH, Mencin AA, Schwabe RF. Toll-like receptor 4 and hepatic fibrogenesis. *Semin Liver Dis*. 2010;30(3):232-244.
4. Heymann F, Trautwein C, Tacke F. Monocytes and macrophages as cellular targets in liver fibrosis. *Inflamm Allergy Drug Targets*. 2009;8(4):307-318.
5. Fallowfield JA, Mizuno M, Kendall TJ, et al. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J Immunol*. 2007;178(8):5288-5295.
6. Duffield JS, Forbes SJ, Constandinou CM, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest*. 2005;115(1):56-65.
7. Ramachandran P, Iredale JP. Macrophages: Central regulators of hepatic fibrogenesis and fibrosis resolution. *J Hepatol*. 2012;56(6):1417-1419.
8. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol*. 2004;4(8):583-594.
9. Guo J, Loke J, Zheng F, et al. Functional linkage of cirrhosis-predictive single nucleotide polymorphisms of toll-like receptor 4 to hepatic stellate cell responses. *Hepatology*. 2009;49(3):960-968.
10. Bates JM, Akerlund J, Mittge E, Guillemin K. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell Host Microbe*. 2007;2(6):371-382.
11. Poelstra K, Bakker WW, Klok PA, Hardonk MJ, Meijer DK. A physiologic function for alkaline phosphatase: Endotoxin detoxification. *Lab Invest*. 1997;76(3):319-327.
12. Poelstra K, Bakker WW, Klok PA, Kamps JA, Hardonk MJ, Meijer DK. Dephosphorylation of endotoxin by alkaline phosphatase in vivo. *Am J Pathol*. 1997;151(4):1163-1169.
13. Tuin A, Poelstra K, de Jager-Krikken A, et al. Role of alkaline phosphatase in colitis in man and rats. *Gut*. 2009;58(3):379-387.
14. Moss AK, Hamarneh SR, Mohamed MM, et al. Intestinal alkaline phosphatase inhibits the proinflammatory nucleotide uridine diphosphate. *Am J Physiol Gastrointest Liver Physiol*. 2013.
15. Krausgruber T, Blazek K, Smallie T, et al. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol*. 2011;12(3):231-238.
16. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958-969.
17. Raes G, De Baetselier P, Noel W, Beschin A, Brombacher F, Hassanzadeh Gh G. Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J Leukoc Biol*. 2002;71(4):597-602.
18. Webb DC, McKenzie AN, Foster PS. Expression of the Ym2 lectin-binding protein is dependent on interleukin (IL)-4 and IL-13 signal transduction: Identification of a novel allergy-associated protein. *J Biol Chem*. 2001;276(45):41969-41976.
19. Rietschel ET, Kirikae T, Schade FU, et al. Bacterial endotoxin: Molecular relationships of structure to activity and function. *FASEB J*. 1994;8(2):217-225.
20. Rietschel ET, Brade L, Schade U, et al. Bacterial lipopolysaccharides: Relationship of structure and conformation to endotoxic activity, serological specificity and biological function. *Adv Exp Med Biol*. 1990;256:81-99.
21. Rietschel ET, Brade L, Brandenburg K, et al. Chemical structure and biologic activity of bacterial and synthetic lipid A. *Rev Infect Dis*. 1987;9 Suppl 5:S527-36.
22. Holst O, Thomas-Oates JE, Brade H. Preparation and structural analysis of oligosaccharide monophosphates obtained from the lipopolysaccharide of recombinant strains of salmonella minnesota and escherichia coli expressing the genus-specific epitope of chlamydia lipopolysaccharide. *Eur J Biochem*. 1994;222(1):183-194.
23. Tuin A, Huizinga-Van der Vlag A, van Loenen-Weemaes AM, Meijer DK, Poelstra K. On the role and fate of LPS-dephosphorylating activity in the

rat liver. *Am J Physiol Gastrointest Liver Physiol*. 2006;290(2):G377-85.

24. Goldberg RF, Austen WG,Jr, Zhang X, et al. Intestinal alkaline phosphatase is a gut mucosal defense factor maintained by enteral nutrition. *Proc Natl Acad Sci U S A*. 2008;105(9):3551-3556.

25. Koyama I, Matsunaga T, Harada T, Hokari S, Komoda T. Alkaline phosphatases reduce toxicity of lipopolysaccharides in vivo and in vitro through dephosphorylation. *Clin Biochem*. 2002;35(6):455-461.

26. Scholtens HB, Hardonk MJ, Meijer DK. A kinetic study of hepatic uptake of canine intestinal alkaline phosphatase in the rat. *Liver*. 1982;2(1):1-13.

27. Beumer C, Wulferink M, Raaben W, Fiechter D, Brands R, Seinen W. Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. *J Pharmacol Exp Ther*. 2003;307(2):737-744.

28. Malo MS, Alam SN, Mostafa G, et al. Intestinal alkaline phosphatase preserves the normal homeostasis of gut microbiota. *Gut*. 2010;59(11):1476-1484.

29. Thurman RG, Bradford BU, Iimuro Y, et al. The role of gut-derived bacterial toxins and free radicals in alcohol-induced liver injury. *J Gastroenterol Hepatol*. 1998;13 Suppl:S39-50.

30. Gabele E, Dostert K, Hofmann C, et al. DSS induced colitis increases portal LPS levels and enhances hepatic inflammation and fibrogenesis in experimental NASH. *J Hepatol*. 2011;55(6):1391-1399.

31. Lee TH, Kim WR, Poterucha JJ. Evaluation of elevated liver enzymes. *Clin Liver Dis*. 2012;16(2):183-198.

32. Laskin DL, Sunil VR, Gardner CR, Laskin JD. Macrophages and tissue injury: Agents of defense or destruction? *Annu Rev Pharmacol Toxicol*. 2011;51:267-288.

33. Hemmann S, Graf J, Roderfeld M, Roeb E. Expression of MMPs and TIMPs in liver fibrosis - a systematic review with special emphasis on anti-fibrotic strategies. *J Hepatol*. 2007;46(5):955-975.

34. Song E, Ouyang N, Horbelt M, Antus B, Wang M, Exton MS. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol*. 2000;204(1):19-28.

35. Cook PC, Jones LH, Jenkins SJ, Wynn TA, Allen JE, Macdonald AS. Alternatively activated dendritic cells regulate CD4+ T-cell polarization in vitro and in vivo. *Proc Natl Acad Sci U S A*. 2012;109(25):9977-9982.

36. Gordon S, Martinez FO. Alternative activation of macrophages: Mechanism and functions. *Immunity*. 2010;32(5):593-604.

37. Krausgruber T, Saliba D, Ryzhakov G, Lanfrancotti A, Blazek K, Udalova IA. IRF5 is required for late-phase TNF secretion by human dendritic cells. *Blood*. 2010;115(22):4421-4430.

38. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol*. 2013;229(2):176-185.

39. Pradere JP, Troeger JS, Dapito DH, Mencin AA, Schwabe RF. Toll-like receptor 4 and hepatic fibrogenesis. *Semin Liver Dis*. 2010;30(3):232-244.

40. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958-969.

CHAPTER 7

General discussion and future perspectives

BIOLOGICALS AND HOMEOSTASIS

Prostaglandins and cytokines regulate all kinds of biological processes in the body[1,2]. This thesis shows that prostaglandins and cytokines are not just 'good' or 'bad' during the progression of fibrogenesis but that they act differently in different phases of disease. Our data show that both PGE_2 and IFNγ stimulate wound healing in the acute phase of liver fibrosis, yet they attenuate liver fibrosis in its chronic phase. This dynamic response of the liver might be the result of a different composition of cells during the acute and chronic phase. In the acute phase, the liver contains many inflammatory cells and a relative low number of activated HSC that are present to repair the damaged areas[3]. Activated HSC and myofibroblasts are much more prominent in the chronic phase of the fibrogenenic process[3,4]. In addition, macrophage polarization also plays a major role. Whereas the M1 macrophage phenotype induces pro-inflammatory effects and secretes matrix degrading MMP's, M2 macrophages induce anti-inflammatory effects and support matrix synthesis[5-7]. So, stimulation of macrophage activity might result in a different outcome when the composition of cells is different. This affects the responses upon biologicals or drugs in the acute and chronic phase of disease.

We found that PGE_2 and IFNγ induce in both phases a Th1 response within the liver resulting in ECM synthesis in the acute phase but ECM degradation in the chronic phase. These opposite effects may be explained by assuming that PGE_2 and IFNγ drive the different repair mechanisms towards homeostasis. Immediately after damage, an inflammatory response towards the inciting agent and effective wound healing are essential to preserve liver function. However, when the pathogenic process has led to a situation of excessive ECM deposition with derangement of the liver architecture, PGE_2 and IFNγ enhance inflammation, reduce fibrogenesis and stimulate ECM degradation, again leading to resolution of damage and thus to homeostasis.

This view may explain many conflicting results on liver fibrosis seen with anti-inflammatory or pro-inflammatory compounds and it could mean in clinical practice that a complete assessment of a patients profile is necessary before starting a treatment. IFNγ has already been tested in several clinical trials in renal-, pulmonary- and liver fibrosis[8-10]. However, its restricted efficacy and high systemic side effects limited its clinical utility. Maybe the restricted efficacy of this very potent cytokine is due to a combination of pro- and antifibrotic effects in a mixed population. Muir *et all* found that only a selected subgroup of fibrotic patients with Hepatitis C responded significantly to the IFNγ therapy[11] and Jeong *et all* suggested that the contradictory results of several IFNγ-based clinical trials in patients with liver fibrosis may be due to differences in degree of fibrosis[12], associated with variations in the activation states of HSC and NK cell influx[12]. This may have important implications for the treatment of liver fibrosis: the effect of cyclo-oxygenase inhibitors or other pro- or anti-inflammatory compounds is determined by the fact whether the disease is in its early or late stage.

Based on this view, it can be deduced that fibrosis might be treated with pro-inflammatory biologicals. Since patients with liver fibrosis are weak at the end-stage and these compounds induce systemic inflammation it is however necessary to deliver these compounds in the target cell. This is the subject of ongoing investigations.

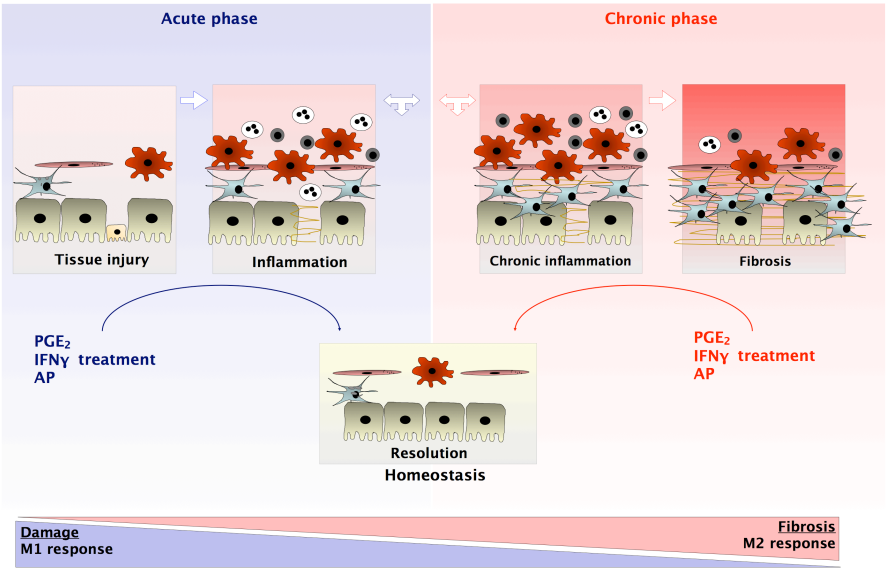

Figure 1. Schematic overview of the effects of PGE_2, IFNγ and AP in the acute and chronic phase after liver damage.

TARGET CELL OF PROSTAGLADIN E_2 IN CHRONIC FIBROGENESIS

In the liver the mechanism behind the effects of PGE_2 has not yet been extensively investigated. PGE_2 is known to have pleiotropic effects and is endowed with a poor pharmacokinetic profile; it is rapidly cleared by the liver and kidneys, inactivated by plasma proteins or metabolized and oxidized in plasma[13]. Despite all this, we found anti-fibrotic effects in livers of mice treated with PGE_2, associated with changes in macrophage polarization and the recently identified cAMP effector Epac-1. Epac-1 proteins are expressed in several cell types including monocytes, macrophages, B and T cells, blood cells and fibroblasts[14]. We used our drug targeting approach (see review[15]) to determine which cell-type was responsible for the Epac-1-mediated effects seen in livers *in vivo*. Only in HSC and not in hepatocytes and KC, PGE_2 treatment inhibited ECM deposition and this was paralleled by changes in the cAMP effector Epac-1. PGE_2 is a lipid mediator with locally a broad range of target cells in the liver[16-23]. Obviously, the composition of cells determines to which cells PGE_2 binds via its EP receptor. In advanced liver fibrosis, inflammatory cell in flux is less prominent compared to accumulation of fibrogenic cells. The excessive amount of HCS present in the fibrotic livers, elicit a response from these cells induced by PGE_2 via EP receptors.
So, drug targeting studies showed that the HSC is responsible for the PGE_2 -induced anti-fibrotic effects seen in a chronic CCl_4 model of liver fibrosis. Although PGE_2 was delivered to the PDGF/receptor on HSC in these studies, we also observed effects on macrophage polarization in these animals. This can be explained by:

1. Indirect effect

The PGE-conjugate binds to the PDGF receptor at the HSC, leading to receptor-mediated internalization and subsequently degradation of the construct in lysosymes. After degradation PGE_2 is released from the

carrier. Its lipophilic nature ensures release from the lysosomal compartment and outer cell membrane and PGE_2 can bind to its EP receptor present on the HSC. The HSC in turn communicates with macrophages by the expression of a scale of chemokines. Recent reports illuminate the nature of this interaction between both cell types[24].

2. Direct effect

Again, the PGE-conjugate binds to the PDGF receptor at the HSC and after degradation the free PGE_2 molecule diffuses over the cell membrane and binds to the EP receptor present on macrophages. The macrophages in turn steer the process by the release of cytokines and chemokines and trigger HSC activation or fibrolysis.
This can not be examined in the present study, but in both cases, local release of PGE_2 from its carrier mimics the physiological situation of its local production.

THE ROLE OF LPS IN LIVER FIBROGENESIS

A well known endogenous mediator that is able to modify the polarization of macrophages is LPS. LPS is derived from the gut and may cross the intestinal wall in particular during liver fibrosis. Studies from our lab have shown that alkaline phosphatase (AP) is able to detoxify LPS[25-27] and it is well known that both serum- as well as intrahepatic levels of AP-activity rise during liver fibrosis. In fact, serum AP is routinely used as a serum marker for many liver diseases including fibrosis, but its role during these diseases is unknown. In intestinal-AP KO mice (iAP KO) we found a significant higher wound repair process which was inhibited by treatment with antibiotics. So, lack of AP in the intestine stimulates the wound healing process in the liver, most likely due to reduced detoxification of LPS. Unfortunately LPS levels are too low to measure *in vivo* to confirm this hypothesis. The increased collagen deposition and HSC-activation was associated with a shift in M1/M2 balance towards M2 macrophages. In general, higher accumulation of M1 macrophages are expected by increased levels of LPS[28,29]. However, it is well known that a Th1 (acute inflammation) response is followed by a Th2 (repair) response[7,30]. In 6 weeks old iAP KO mice the LPS-triggered inflammatory state is apparently followed by such a Th2 repair response. In time, collagen deposition may increase although no reports indicate that this KO-model is associated with a fibrotic liver. Most likely, more triggers are needed to reach such a state.

The M1 cells rapidly and massively respond to an inciting stimulus. These cells degrade the extracellular matrix and to prevent a self-destructing action in the organ, a strong counterbalance by M2 cells is needed to reduce inflammation, repair the damaged areas and encapsulate the source. We found that AP fulfills protective actions in acute and chronic fibrogenesis and steers the process towards homeostasis. In the acute phase, exogenous AP stimulates wound healing combined with a high M2 accumulation. In this phase AP may inhibit inflammation by the detoxification of LPS and stimulate repair of the necrotic areas. Increased permeability of the intestinal wall and a higher uptake of LPS, aggravates liver damage and fibrosis[31-33]. During chronic fibrogenesis, higher levels of hepatic AP were found in the fibrotic bands and administration of iAP resulted in a reduced collagen deposition associated with lower accumulation of M2. Apparently, detoxification of LPS by AP inhibits M1 macrophage triggering and thereby also the subsequent M2 response.

CONCLUSIONS

In general, our results, together with several other studies[30,34-36] indicate that there is a tight balance between HSC activation and macrophage polarization. Although HSC and fibroblasts are the main producers of collagens, the balance between M1 and M2 macrophages also greatly determines disease progression or regression in the liver. The human body is often exposed to inflammatory factors that lead to a fulminant reaction to eliminate the intruder. These inflammatory processes are in general immediately counterbalanced by M2-mediated processes. Small changes in M1 macrophages may lead to enormous changes in M2 macrophages (see chapter 2, 3 and 6, this thesis). M2 macrophages orchestrate processes that directly encapsulate the inciting stimulus, stop the destroying inflammatory response and repair the damaged areas and protect the body. Only when the trigger exists for many years an excessive M2 response arises and a deregulated balance between collagen synthesis and degradation leads to ruination of the liver architecture and its function. However, in case of liver diseases, such a total deregulation of liver function may take up to three decades, which reflects the power of this balance.

REFERENCES

1. Aoki T, Narumiya S. Prostaglandins and chronic inflammation. *Trends Pharmacol Sci*. 2012;33(6):304-311.
2. Bansal R, Prakash J, Post E, Beljaars L, Schuppan D, Poelstra K. Novel engineered targeted interferon-gamma blocks hepatic fibrogenesis in mice. *Hepatology*. 2011;54(2):586-596.
3. Heymann F, Trautwein C, Tacke F. Monocytes and macrophages as cellular targets in liver fibrosis. *Inflamm Allergy Drug Targets*. 2009;8(4):307-318.
4. Friedman SL. Hepatic fibrosis -- overview. *Toxicology*. 2008;254(3):120-129.
5. Barron L, Wynn TA. Fibrosis is regulated by Th2 and Th17 responses and by dynamic interactions between fibroblasts and macrophages. *Am J Physiol Gastrointest Liver Physiol*. 2011;300(5):G723-8.
6. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol*. 2012.
7. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol*. 2004;4(8):583-594.
8. Pockros PJ, Jeffers L, Afdhal N, et al. Final results of a double-blind, placebo-controlled trial of the antifibrotic efficacy of interferon-gamma1b in chronic hepatitis C patients with advanced fibrosis or cirrhosis. *Hepatology*. 2007;45(3):569-578.
9. Oldroyd SD, Thomas GL, Gabbiani G, El Nahas AM. Interferon-gamma inhibits experimental renal fibrosis. *Kidney Int*. 1999;56(6):2116-2127.
10. King TE,Jr, Albera C, Bradford WZ, et al. Effect of interferon gamma-1b on survival in patients with idiopathic pulmonary fibrosis (INSPIRE): A multicentre, randomised, placebo-controlled trial. *Lancet*. 2009;374(9685):222-228.
11. Muir AJ, Sylvestre PB, Rockey DC. Interferon gamma-1b for the treatment of fibrosis in chronic hepatitis C infection. *J Viral Hepat*. 2006;13(5):322-328.
12. Jeong WI, Park O, Suh YG, et al. Suppression of innate immunity (natural killer cell/interferon-gamma) in the advanced stages of liver fibrosis in mice. *Hepatology*. 2011;53(4):1342-1351.
13. Hamberg M, Samuelsson B. On the metabolism of prostaglandins E 1 and E 2 in man. *J Biol Chem*. 1971;246(22):6713-6721.
14. Roscioni SS, Elzinga CR, Schmidt M. Epac: Effectors and biological functions. *Naunyn Schmiedebergs Arch Pharmacol*. 2008;377(4-6):345-357.
15. Poelstra K, Prakash J, Beljaars L. Drug targeting to the diseased liver. *J Control Release*. 2012;161(2):188-197.
16. Hirata T, Narumiya S. Prostanoids as regulators of innate and adaptive immunity. *Adv Immunol*. 2012;116:143-174.

17. Hirata T, Narumiya S. Prostanoid receptors. *Chem Rev*. 2011;111(10):6209-6230.

18. Rincon-Sanchez AR, Covarrubias A, Rivas-Estilla AM, et al. PGE2 alleviates kidney and liver damage, decreases plasma renin activity and acute phase response in cirrhotic rats with acute liver damage. *Exp Toxicol Pathol*. 2005;56(4-5):291-303.

19. Mallat A, Gallois C, Tao J, et al. Platelet-derived growth factor-BB and thrombin generate positive and negative signals for human hepatic stellate cell proliferation. role of a prostaglandin/cyclic AMP pathway and cross-talk with endothelin receptors. *J Biol Chem*. 1998;273(42):27300-27305.

20. Ruwart MJ, Wilkinson KF, Rush BD, et al. The integrated value of serum procollagen III peptide over time predicts hepatic hydroxyproline content and stainable collagen in a model of dietary cirrhosis in the rat. *Hepatology*. 1989;10(5):801-806.

21. Weinberg E, Zeldich E, Weinreb MM, Moses O, Nemcovsky C, Weinreb M. Prostaglandin E2 inhibits the proliferation of human gingival fibroblasts via the EP2 receptor and epac. *J Cell Biochem*. 2009;108(1):207-215.

22. Haag S, Warnken M, Juergens UR, Racke K. Role of Epac1 in mediating anti-proliferative effects of prostanoid EP(2) receptors and cAMP in human lung fibroblasts. *Naunyn Schmiedebergs Arch Pharmacol*. 2008;378(6):617-630.

23. Huang S, Wettlaufer SH, Hogaboam C, Aronoff DM, Peters-Golden M. Prostaglandin E(2) inhibits collagen expression and proliferation in patient-derived normal lung fibroblasts via E prostanoid 2 receptor and cAMP signaling. *Am J Physiol Lung Cell Mol Physiol*. 2007;292(2):L405-13.

24. Puche JE, Lee YA, Jiao J, et al. A novel murine model to deplete hepatic stellate cells uncovers their role in amplifying liver damage. *Hepatology*. 2012.

25. Poelstra K, Bakker WW, Klok PA, Hardonk MJ, Meijer DK. A physiologic function for alkaline phosphatase: Endotoxin detoxification. *Lab Invest*. 1997;76(3):319-327.

26. Poelstra K, Bakker WW, Klok PA, Kamps JA, Hardonk MJ, Meijer DK. Dephosphorylation of endotoxin by alkaline phosphatase in vivo. *Am J Pathol*. 1997;151(4):1163-1169.

27. Tuin A, Huizinga-Van der Vlag A, van Loenen-Weemaes AM, Meijer DK, Poelstra K. On the role and fate of LPS-dephosphorylating activity in the rat liver. *Am J Physiol Gastrointest Liver Physiol*. 2006;290(2):G377-85.

28. Krausgruber T, Saliba D, Ryzhakov G, Lanfrancotti A, Blazek K, Udalova IA. IRF5 is required for late-phase TNF secretion by human dendritic cells. *Blood*. 2010;115(22):4421-4430.

29. Laskin DL, Sunil VR, Gardner CR, Laskin JD. Macrophages and tissue injury: Agents of defense or destruction? *Annu Rev Pharmacol Toxicol*. 2011;51:267-288.

30. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol*. 2013;229(2):176-185.

31. Enomoto N, Ikejima K, Yamashina S, et al. Kupffer cell sensitization by alcohol involves increased permeability to gut-derived endotoxin. *Alcohol Clin Exp Res*. 2001;25(6 Suppl):51S-4S.

32. Enomoto N, Ikejima K, Bradford BU, et al. Role of kupffer cells and gut-derived endotoxins in alcoholic liver injury. *J Gastroenterol Hepatol*. 2000;15 Suppl:D20-5.

33. Pradere JP, Troeger JS, Dapito DH, Mencin AA, Schwabe RF. Toll-like receptor 4 and hepatic fibrogenesis. *Semin Liver Dis*. 2010;30(3):232-244.

34. Ramachandran P, Iredale JP. Macrophages: Central regulators of hepatic fibrogenesis and fibrosis resolution. *J Hepatol*. 2012;56(6):1417-1419.

35. Duffield JS, Forbes SJ, Constandinou CM, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest*. 2005;115(1):56-65.

36. Fallowfield JA, Mizuno M, Kendall TJ, et al. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J Immunol*. 2007;178(8):5288-5295.

CHAPTER 8

Summary

Liver fibrosis is a multi-cellular process characterized by an abundant deposition of extracellular matrix (ECM) resulting in hepatocellular dysfunction and finally death. Up to now this disease is untreatable, which is partly due to complex cell-cell interactions during the fibrogenic process, and many positive and negative feedback loops that make therapeutic interventions troublesome. After liver injury by for instance toxins, autoimmune diseases, drugs or alcohol, damaged hepatocytes activate resident macrophages (kupffer cells, KC) that, together with recruited macrophages, in turn secrete many other cytokines and growth factors. These growth factors including Transforming Growth Factor-β (TGF-β) activate the hepatic stellate cell (HSC), that produce ECM constituents. It has become clear that macrophages play a key role in the activation and regulation of HSC, but the regulatory mechanisms are largely unknown.

Macrophages can be divided into at least two subtypes; the pro-inflammatory M1 macrophage and the M2 macrophage with wound-healing capacities. Nowadays, the role and accumulation of both subtypes during liver fibrogenesis is unknown. We therefore first studied the localization and the M1/M2 composition during acute and chronic fibrogenesis, as described in **chapter 2**. During fibrogenesis in mice and human livers, macrophages migrate from the parenchymal area to the fibrotic lesions. While in the acute phase a balanced increment of both macrophages subtypes was found, in the chronic phase this balance was shifted towards the pro-fibrotic M2 macrophage. In contrast, during liver regeneration, the M1/M2 balance shifted to the MMP-producing M1 macrophage. The M2 macrophages were completely absent. These changes in macrophage composition during disease progression and reversal might implicate the importance of the M1/M2 balance and manipulation of this balance may provide new leads for therapy. We therefore studied the effects of endogenous mediators prostaglandin E_2 (PGE_2) and interferon-γ (IFNγ) on macrophage polarization and fibrogenic parameters during fibrogenesis, as described in **chapter 3**. Both mediators are produced by many cell types within the fibrotic liver and, most importantly, they may regulate different cell types involved in the fibrotic process. We found that PGE_2 and IFNγ both affected the polarisation of macrophages *in vivo*, thereby stimulating repair mechanisms in the early phase and fibrolysis in the late phase of disease, steering the process towards homeostasis in both conditions.

The next step was to unravel the intracellular pathway of the cyclo-oxygenase product PGE_2 *in vivo*. **Chapter 4** describes the role of cAMP-mediator exchange protein activated by cAMP-1 (Epac-1) in liver fibrosis. We found that fibrogenesis was associated with a reduced Epac-1 expression in mice and human liver samples. Treatment of fibrotic mice with cAMP-activator PGE_2 normalized these Epac-1 expression levels and attenuated fibrogenesis. Opposite effects were found after treatment with the COX2 inhibitor Niflumic acid (NFA). Detailed insight in this process is essential to understand the long term effects of COX inhibitors during fibrogenesis, which is relevant to millions of people world-wide. The effects of PGE_2 are complex: it regulates multiple (patho)physiological processes by activation or inhibition of cAMP activity via several receptors (EP1-4), which are expressed on a variety of cells. In **chapter 5** we aimed to investigate which cell type is responsible for the PGE_2-mediated effects on fibrosis that were seen *in vivo*. A significant correlation between intrahepatic Epac-1 expression and collagen deposition *in vivo* was found in fibrotic mice. Cell-specific delivery studies identified the hepatic stellate cell as the key cell for the anti-fibrotic effects of PGE_2. HSC-directed PGE_2 normalized the hepatic Epac-1 expression levels, which was associated with reduced ECM deposition. Delivery of PGE_2 to other hepatic cell types did not induce these effects. In summary, PGE_2 induced anti-fibrotic effects in mice with advanced

liver fibrosis which was associated with (direct or indirect) alterations in HSC-specific Epac-1 levels and changes in macrophage polarization. This provides evidence for a complex interaction between HSC and macrophages in the liver.

The liver serves as the main clearance mechanism for particles in the blood and therefore contains the majority of macrophages present in the body. In addition, the liver is the first-pass organ for gut derived toxins and foreign particles including lipopolysaccharide (LPS). Gut-derived LPS is known to aggravate liver damage and fibrosis and LPS is also associated with the polarization of macrophages into the M1 subtype. In **chapter 6** we therefore studied the role of intestinal alkaline phosphatase (iAP), during fibrogenesis. This enzyme attenuates the toxicity of LPS by dephosphorylation of the toxic part of the LPS molecule (i.e. the lipid A moiety). We found that exogenous alkaline iAP attenuated fibrosis significantly. This was associated with reduced levels of M2 macrophages within livers of iAP-treated fibrotic mice. In livers of mice and man an enhanced expression of AP activity was found in fibrotic septa, and we showed that the enzyme was able to dephosphorylate LPS. This enhanced expression of AP in fibrotic livers and the attenuated fibrogenesis in fibrotic mice receiving iAP suggests that endogenous AP serves as a protective enzyme after liver damage. This protective effect was confirmed by increased wound healing activities observed in mice with acute liver damage after administration of iAP. Upregulation of this enzyme activity may represent another regulatory mechanism that modifies macrophage activity and thereby fibrogenesis.

Collectively, the studies presented in this thesis demonstrate the complex interactions between hepatic stellate cells and macrophages that can polarize into M1 and M2 subtypes and indicate that PGE_2, IFNγ, LPS and AP are important actors in this fibrotic scene.

CHAPTER 9

Samenvatting voor de leek

De lever is een complex orgaan en vervult velerlei functies in het lichaam. Zo is de lever betrokken bij de bloedstolling, de stofwisseling van koolhydraten, vetten en eiwitten en is het meest belangrijke orgaan bij het ontgiften van lichaamsvreemde of toxische stoffen uit het bloed. De lever heeft een grote regeneratieve capaciteit wat betekent dat dit orgaan in staat is zichzelf volledig te herstellen na schade veroorzaakt door toxische stoffen zoals alcohol, virussen, vetten en sommige geneesmiddelen. Echter, langdurige blootstelling van de lever aan deze schadelijke prikkels kan resulteren in leverfibrose met als eindstadium cirrose. Leverfibrose is een chronische aandoening die wordt gekarakteriseerd door een verstoord regeneratie proces, zich uitend in een excessieve accumulatie van littekenweefsel (collageen) in de lever. Deze grote hoeveelheden collageen verstoren de structuur van de lever en hiermee ook de functionaliteit. Het proces dat hieraan ten grondslag ligt is complex en het duurt vaak jaren voordat de ziekte klinisch tot uiting komt. Vaak is op het moment van diagnose het wegnemen van de oorzaak niet meer voldoende om deze progressieve ziekte te stoppen wat uiteindelijk zal leiden tot de dood. Tot op heden is er geen therapie beschikbaar die het proces effectief remt of omkeerbaar maakt en is het uitvoeren van een lever transplantatie de enige mogelijkheid, hetgeen grote complicaties met zich mee kan brengen en niet geschikt is voor iedere patiënt.

Het ontstaan van leverfibrose is een complex proces omdat er veel cellen bij betrokken zijn en kan globaal worden ingedeeld in een acute en een chronische fase. Acute, eenmalige schade aan de hepatocyt, de functionele cel van de lever, zorgt voor vrijmaking van ontstekingsmediatoren wat resulteert in een influx van ontstekingscellen en activatie van macrofagen. Deze cellen scheiden op hun beurt weer een scala aan mediatoren uit en activeren hiermee de hepatische stellaat cell (HSC). Deze geactiveerde HSC nemen in aantal toe en ze veranderen in myofibroblast-achtige cellen. Deze produceren collagenen en repareren hiermee de beschadigde stukken in de lever. Wanneer de beschadiging chronisch wordt, neemt het aantal myofibroblast-achtige cellen in de lever sterk toe en worden dikke collageen bundels gevormd. De afgelopen jaren hebben onderzoekers voornamelijk geprobeerd om geactiveerde HSC te remmen en hiermee de collageen depositie tegen te gaan. Verschillende geneesmiddelen hebben zowel *in vitro* als *in vivo* mooie anti-fibrotische effecten laten zien maar in de kliniek heeft dit helaas nooit geleid tot een goede, werkzame therapie. De laatste tijd is men zich echter ervan bewust geworden dat naast de HSC, de macrofaag een prominente plaats inneemt bij de ontwikkeling van het ziekteproces.

Macrofagen vervullen een cruciale rol bij de eerste afweer van een organisme, maar zijn ook nauw betrokken bij de regulatie van regeneratie processen en weefsel herstel. Om deze verschillende processen uit te voeren zijn macrofagen globaal in te delen in twee subtypen; de klassieke geactiveerde macrofaag (M1) en de alternatief geactiveerde macrofaag (M2). Beide macrofaag typen worden geactiveerd door verschillende mediatoren en vervullen verschillende activiteiten. M1 macrofagen worden geactiveerd door pro-inflammatoire mediatoren en zijn vooral actief in de acute fase van een ontstekingsproces. Zij induceren een heftige ontstekingsreactie om binnengedrongen schadelijke stoffen of virussen te elimineren. M1 macrofagen produceren collageen-afbrekende enzymen, een soort weekmakers, om de ontstekingscellen door het weefsel heen op de plaats van bestemming te krijgen. Om de beschadigde gebieden te repareren induceert het lichaam vervolgens een tegenrespons waar de M2 macrofagen bij betrokken zijn. M2 macrofagen worden geactiveerd door anti-inflammatoire mediatoren en activeren op hun beurt weer fibroblasten (of HSC) welke collagenen gaan uitscheiden om zo de beschadigde gebieden te repareren. De balans tussen de collageen-afbrekende M1 macrofagen en de collageen-op-

bouwende M2 macrofagen komt dus bijzonder nauw en maakt beide macrofaag subtypen bijzonder interessant om te bestuderen tijdens fibrogenese.

Tot op heden is er nog niets bekend over de rol en de accumulatie van beide macrofaag subtypen tijdens het fibrogenese proces. We zijn daarom begonnen met het in kaart brengen van de lokalisatie en samenstelling van de macrofaag populatie (M1 versus M2) in een begin (acuut) en vergevorderd (chronisch) stadium van leverfibrose, zoals weergegeven in **hoofdstuk 2**. In levers van muis en mens observeerden we een verschuiving van macrofaag accumulatie gedurende fibrose: in normale levers waren de macrofagen gesitueerd in het parenchymale gedeelte van de lever terwijl in fibrotische levers de macrofagen ophoopten in de collageen banden. Door middel van histochemische kleuringen is het ons gelukt om de M1 en M2 macrofagen van elkaar te onderscheiden zodat we de compositie en lokalisatie van beide subtypen in de lever nader konden bestuderen. In het acute stadium was een verhoogde accumulatie van beide macrofaag subtypen waarneembaar in de beschadigde gebieden in de lever. In de chronische fase verschoof deze balans richting de pro-fibrotische M2 macrofagen, gepositioneerd in de collageenbanden. Daarnaast zagen we een significante verschuiving van de M1/M2 balans richting de collageen-afbrekende M1 macrofagen in een regressie model waarin de ziekmakende prikkel werd gestopt. Dit was gekoppeld aan een significante afname van collageen afzetting in deze levers. Deze resultaten laten zien dat de balans tussen de M1 en M2 macrofagen sterk verandert tijdens verschillende fases van het ziekteproces. Deze cellen spelen dus mogelijk een belangrijke rol tijdens fibrogenese en regressie processen en manipulatie van deze balans kan dus van grote therapeutische waarde zijn.

In **hoofdstuk 3** bestuderen we de effecten van Prostaglandine E_2 (PGE_2) en Interferon gamma (IFNγ) op de M1/M2 balans gedurende fibrogenese. Beide endogene mediatoren worden geproduceerd door verschillende cellen die aanwezig zijn in de fibrotische lever en reguleren mogelijk het fibrose proces op verschillende manieren. Zowel PGE_2 als IFNγ behandeling van muizen met acute lever schade liet een toename in reparatie mechanismen zien, samengaand met een significante stijging van het aantal M2 macrofagen. In tegenstelling tot in de acute fase zorgden PGE_2 en IFNγ in muizen met chronische leverfibrose juist voor afbraak van collageen wat geassocieerd was met een daling van het aantal M2 macrofagen. Zowel PGE_2 als IFNγ beïnvloeden dus de macrofaag polarisatie *in vivo* en sturen het proces richting herstel van de gezonde situatie (homeostase).

We hebben dus nu gevonden dat PGE_2 een effect heeft op het fibrose proces en dat de macrofaag compositie verandert na behandeling met PGE_2. We weten echter nog niet of dat effect direct op de macrofagen heeft plaatsgevonden of dat het via een ander cel type in de lever is gegaan (indirect). Om deze reden hebben we getracht de intra-cellulaire signaleringsroute van PGE_2 te ontrafelen *in vivo*. Al vele jaren is bekend dat PGE_2 de cAMP-signaaltransductie in cellen activeert. Deze cAMP-signaaltransductie verloopt via cAMP-effectoren. Recentelijk is naast de welbekende cAMP-effector Protein Kinase A (PKA) een nieuwe signaal transductie route ontdekt, namelijk cAMP-mediator exchange protein activated by cAMP-1 (Epac-1). In **hoofdstuk 4** wordt de rol van Epac-1 en PKA tijdens het fibrogenese proces in de lever beschreven. In fibrotische levers van muis en mens vonden wij significant verlaagde Epac-1 expressie levels in vergelijking met normale, gezonde levers. PGE_2 behandeling van deze muizen zorgde voor normalisatie van deze Epac-1 levels en remde daarbij significant de collageen productie en hiermee dus het fibrose proces. PGE_2 is een mediator die ontstaat door activiteit van het enzym cyclooxygenase (COX). COX-remmers, zoals de welbekende aspirines, worden in de kliniek maar ook thuis veel gebruikt. Behandeling van muizen met leverfibrose met de specifieke COX-2 remmer Nifluminezuur liet, zoals

voorspeld, een tegengesteld effect zien aan PGE_2 op de Epac-1 en collageen depositie in de lever: de Epac-1 expressie werd nog verder verlaagd door deze COX-2 remmer en significant meer fibrotische collageen banden waren waarneembaar. Wereldwijd worden COX-remmers veelvuldig gebruikt en om de lange termijn effecten van deze geneesmiddelen gedurende leverfibrose nader te bepalen is meer gedetailleerde kennis nodig.

PGE_2 reguleert vele (patho)fysiologische processen door manipulatie van cAMP activiteit via binding aan haar EP-receptoren, aanwezig op een scala aan cellen. In **hoofdstuk 5** proberen we te onderzoeken welk cel type in de lever verantwoordelijk is voor de PGE-gemedieerde effecten *in vivo*. Om antwoord op deze vraag te krijgen hebben we gebruik gemaakt van drug-targeting. Het drug targeting concept wordt gebruikt om een stof, in dit geval PGE_2, specifiek af te leveren in de doelcel met behulp van een dragermolecuul (carrier). Deze carrier bestaat uit een eiwit, humaan serum albumine (HSA) waaraan een receptorbindende structuur is gekoppeld. Deze receptorbindende structuren zijn zo ontwikkeld dat zij nagenoeg alleen binden aan de te onderzoeken doelcel. Door vervolgens een geneesmiddel of ander stofje aan dit drager molecuul te koppelen kan het geneesmiddel heel specifiek in de desbetreffende doelcel afgeleverd worden. Wij hebben PGE_2 gekoppeld aan carriers specifiek gericht tegen de HSC, de Kupffer cel (KC, macrofaag van de lever) en de hepatocyt en vervolgens het farmacologisch effect van PGE_2 in de lever bestudeerd. Onze studies lieten zien dat de HSC en niet de KC of de hepatocyt, verantwoordelijk is voor de Epac-1 gemedieerde anti-fibrotische effecten die werden gevonden na PGE_2 toediening.

Naast het detoxificeren van vreemde partikels uit het bloed is de lever ook het eerste en belangrijkste orgaan dat toxines die vanuit de darm in de bloedbaan komen, zoals lipopolysaccharide (LPS), onschadelijk kan maken. LPS is afkomstig van bacteriën uit de darm en is een potente activator van ontstekingscellen. Normaal gesproken "lekken" er kleine hoeveelheden LPS uit de darm en is de lever in staat deze te detoxificeren zonder enige significante ontstekingsreactie. Indien er echter chronisch grotere hoeveelheden LPS uit de darm lekken wordt op den duur het ontstekingssysteem toch geactiveerd met leverschade als gevolg. Zoals eerder beschreven spelen de macrofagen een grote rol in de acute en chronische fases van het fibrogenese proces. LPS stimuleert de polarisatie van macrofagen naar het pro-inflamatoire M1 subtype, een proces dat wordt opgevolgd door de accumulatie van de anti-inflammatoire M2 macrofagen. Er is dus een grote kans dat de M1/M2 balans in de lever zal veranderen na herhaaldelijke blootstelling aan LPS. In **hoofdstuk 6** bestuderen we daarom de rol van alkalisch fosfatase (AP), een enzym dat in staat is LPS onschadelijk te maken door een fosfaat groep van LPS te halen. Significant verhoogde expressie van AP was gevonden in de collageen banden van fibrotische muizen levers in vergelijking met normale, gezonde levers. Daarbij resulteerde de behandeling van fibrotische muizen met het LPS-detoxificerend enzym AP in een verminderde collageen depositie, samengaand met verlaagde expressie levels van de collageen-producerende M2 macrofagen. Muizen die het AP enzym misten (AP-knock out muizen) lieten het omgekeerde zien: in levers van deze muizen werden meer M2 macrofagen en een verhoogde collageen productie gevonden ten opzichte van normale muizen. Deze resultaten suggereren dus een beschermende rol van AP tijdens fibrogenese.

Samenvattend kan gezegd worden dat de studies gepresenteerd in dit proefschrift de complexe interacties tussen de HSC en macrofagen laat zien. De samenstelling van cellen (HSC, M1 en M2 macrofagen) verandert sterk in de lever gedurende de verschillende stadia van het fibrose proces en daarmee veranderen eveneens de effecten van PGE_2 en IFNγ. Ook LPS en AP beïnvloeden dit proces en met name de rol van deze laatste acteur was onbekend tijdens leverfibrose. De verschillende macrofaag subtypen en

biologicals zoals PGE_2, IFNγ, LPS en AP blijken belangrijke spelers in het fibrogenese proces in de lever en het is dus het decor dat de rol van deze spelers bepaalt.

Dankwoord

Voordat ik aan mijn PhD begon had ik niet verwacht dat deze baan zoveel meer zou omvatten dan het behalen van onderzoeksresultaten alleen. De leercurve is steil geweest op zowel wetenschappelijk- als persoonlijk gebied. Het vergt veel tijd, energie en vasthoudendheid om naast alle deadlines en gestrande experimenten telkens je doelen bij te stellen en te blijven geloven in die mooie hypothese. Ik ben intens dankbaar dat ik zoveel heb mogen leren in een voor mij veilige en warme omgeving. Dit proefschrift zou nooit tot stand zijn gekomen zonder de hulp van de vele mensen om mij heen die zich over mij ontfermd hebben, hetzij met vakinhoudelijke kennis, met praktische hulp, met goede discussies of gewoon met gezellige afleiding. Graag wil ik iedereen hiervoor bedanken, een aantal mensen in het bijzonder.

Allereerst gaat mijn dank uit naar mijn promotor **Klaas Poelstra**. Klaas, het was fijn om jou als directe begeleider te hebben. Ik heb ontzettend veel van je geleerd. De vrijheid die jij mij gegeven hebt om mij mijn eigen koers te leren varen waardeer ik enorm, al waren er, zoals je weet, enkele momenten dat ik diezelfde vrijheid heb vervloekt. Toch moet ik (achteraf) toegeven dat dit misschien wel de beste leerschool is naar zelfstandigheid, creativiteit en probleemoplossend vermogen. Ik wil je graag bedanken voor je vakinhoudelijke hulp, je geduld en flexibiliteit, je adviezen en pep-talks, en zeker ook voor je gezelligheid en openheid. Maar het meest dankbaar ben ik je voor het feit dat je vanaf het begin tot het eind in mij hebt geloofd en mij hebt gecoacht in het ontdekken van mijn valkuilen en talenten.

Mijn dank gaat uit naar **Leonie Beljaars**, mijn co-promotor. Leonie, ook jij hebt een grote rol gespeeld bij het tot stand komen van dit proefschrift. Bedankt voor je inhoudelijke inbreng, je luisterend oor maar ook praktische hulp. Vooral in de eerste jaren was jij degene die de vertaalslag voor mij maakte van een idee naar een experiment. Jij hebt mij altijd gesteund in het 'verkopen' van mijn ideeën naar Klaas toe. Dankjewel daarvoor. Ik heb bewondering voor de keuzes die je hebt gemaakt in je carrière en op jouw ervaring baseer ik nu mijn eigen keuzes.

Ik wil de leden van de leescommissie, **prof. H.J. Haisma, prof. H. van Goor** en **prof. M. Schmidt** bedanken voor de snelle beoordeling van mijn proefschrift. Hidde, ik ben je al vaak tegengekomen in mijn studententijd en ik vind het bijzonder leuk dat je nu getuige bent van mijn promotie. Martina, bedankt voor onze prettige samenwerking. Ondanks je drukke werkzaamheden heb je altijd klaargestaan voor het beantwoorden van mijn vragen en het geven van feedback.

Graag wil ik **Jai Prakash** bedanken. Jai, bedankt voor je hulp bij het 'designen' van mijn constructen en je waardevolle input in discussies. Ik heb veel van je geleerd. Ik wens je zowel op je werk als privé alle goeds toe in Enschede.

Mijn dank gaat uit naar **Barbro Melgert**. Barbro, wat ben ik blij dat jij op de afdeling gekomen bent! Jouw enthousiasme en frisse kijk op het onderzoek werken aanstekelijk. Daarnaast heb je ook nog eens gezorgd voor twee leuke AIO's. Bedankt voor het delen van je kennis, je kritische blik op resultaten, je apple-helpdesk-service en je heerlijke, vezelrijke bakkunsten. Succes met je professor-inwording-traject.

I would like to express my special gratitude to **dr.Sophie Lotersztajn**. Sophie, during my master and PhD, I have been a guest in your lab. Although not all the work has materialized into papers (yet), I

have learned a lot from you. The way you manage the group, perform science at a high level and be mother at the same time has inspired me. I appreciated your hospitality, all the help I got and the pleasant times in beautiful Paris.

Dankzij **Geny Groothuis** ben ik in aanraking gekomen met de vakgroep. Geny, als student assistent heb je mij geïntroduceerd in de vakgroep en daarna ben ik (bijna) niet meer weggeweest. En daar ben ik blij mee! Al ben ik niet een van jouw AIO's geworden toch heb je altijd voor mij klaar gestaan. Bedankt voor je wetenschappelijke input, je warme persoonlijkheid, interesse en begrip.

Mijn dank gaat uit naar de analisten die altijd hebben klaargestaan om mij zowel praktisch als persoonlijk te ondersteunen. **Eduard**, mijn maatje, bij alle resultaten weergegeven in dit boekje ben jij, in grote of in kleine mate, betrokken geweest. Ik weet niet hoe ik je moet bedanken voor al het werk dat je verzet hebt. Zonder morren heb je hebt altijd klaargestaan om het onderzoek goed te laten verlopen; of het nu weekend was of doordeweeks, vakantie of geen vakantie, 6.30 of 23.00 uur, jij was er gewoon! Jij hebt een grote bijdrage geleverd aan de betrouwbare en veilige basis die ik nodig had om te kunnen leren en presteren. Heel erg bedankt voor je flexibiliteit, steun, begrip, gezelligheid en je kookkunsten. Fijn dat je m'n paranimf bent. Ik wens je alle goeds toe samen met je lieve vrouw en kids! **Catharina**, jij bent een stabiele, betrouwbare en waardevolle spil op het lab. Al vanaf mijn studententijd loop ik de deur bij je plat voor hulp of gewoon een gezellig kletsje. Erg bedankt voor je protocollen, tips & trucs, luisterend oor, gezelligheid en alle experimenten die je voor mij gedaan hebt. **Alie**, jij bent met stip de meest kleurrijke verschijning van het lab geweest. Zo uitgesproken dat je kleding is zo uitgesproken is ook jouw gevoelsleven. Dankjewel voor je medeleven, raad, gezelligheid en stralende lach! Sinds jouw vertrek is het een stuk stiller geworden op het lab. **Edwin**, roomie voor een jaar; bedankt voor je humor en gezelligheid! **Marieke**, ook jij bedankt voor je gezelligheid op het lab en tijdens etentjes.

Mijn lieve mede AIO's / lotgenoten wil ik graag bedanken. **Marike**, vanwege jouw voorsprong op het AIO-schap heb ik altijd alles een beetje af kunnen kijken. Bedankt voor al je hulp, goede gesprekken, gezelligheid en gastvrijheid. Het was fijn dat ik bij jou in huis mocht wonen toen ik moest knallen voor mijn onderzoek en de reisafstand te groot werd. Succes met je carrière als onderzoeker! **Marianne**, ik kan me nog precies herinneren dat je op de afdeling kwam en wat je aanhad (maar dat laatste is mijn tic). Ook al zijn we twee totaal verschillende mensen het klikte meteen. Bedankt voor je gezelligheid, eerlijkheid en openheid. Ik heb moeite gehad met jouw vertrek maar de opleiding die je nu doet past je helemaal! Heel veel succes en plezier! **Ruchi**, ik heb nog nooit iemand ontmoet die zo gedreven is als jij en daar heb ik bijzonder veel respect voor. Bedankt voor je mooie verhalen over de Indiaase cultuur, je gastvrijheid in Groningen en Stockholm, en je advies en hulp bij mijn onderzoek. Geniet van je prachtige gezinnetje en vergeet niet af en toe stil te staan om te realiseren hoeveel je al voor elkaar gebokst hebt. **Adriana**, girl you rocked the last couple of months! Thank you for your support and the dinners we had together. Good luck finishing up your thesis!

En dan 'the new generation' AIO's; Carian, Christina, Karin en Amirah met 'the new generation' post-doc Irma. Met jullie komst is er, naar mijn mening, een flinke boost positieve energie op de afdeling gekomen. Met veel plezier kijk ik terug op onze thema-borrels en etentjes. Jullie hebben

ervoor gezorgd dat ik de laatste maanden van mijn onderzoek met een fijn gevoel naar mijn werk ging terwijl ik mijn gezin achter liet in Ede. **Carian**, op de een of andere manier snap ik jou gewoon. Aan een half woord hebben we genoeg om tot de kern te komen, in een deuk te liggen of een gevoel te uiten. Ik denk met veel plezier terug aan onze fijne gesprekken en natuurlijk het ski-stone avontuur! Dankjewel daarvoor. Heel veel succes en plezier met je onderzoek! **Christina**, het dromerige prinsesje van de afdeling. Met veel verwondering heb ik altijd toegekeken hoe jij rustig iedereen helpt terwijl daardoor je eigen planning in het 100 loopt en je, als gevolg daarvan, tot laat bezig bent met je eigen experimenten. Ik wil je bedanken voor je hulp bij de interpretatie van mijn resultaten, je geduld maar bovenal gezelligheid. Heel veel succes met je onderzoek en je hardloopprak-tijken! **Karin**, bedankt voor je interesse, steun en gezelligheid! Eerst saampjes en later met Amirah, waren wij de AIO's van Klaas aanwezig op de afdeling. Toch besef ik me maar al te goed dat ik weinig tijd heb gehad om jou te 'helpen' opstarten. Sorry daarvoor. Gelukkig dartelde de altijd vrolijke Irma om je heen en heb je nu alle hulp van Eduard en Catharina. Heel veel succes met je onderzoek, het gaat helemaal goed komen! **Amirah**, Good luck with your PhD! **Irma**, jij bent echt de vrolijkste post-doc die ik ooit heb gezien! Bedankt voor je gezelligheid, lachsessies en positieve energie. Het is een stuk stiller geworden sinds je vertrek. Succes met je opleiding tot Klinisch Chemicus. Super stoer!

Ik heb het geluk gehad om vier fantastische master-studenten te mogen begeleiden. **Irene**, **Stijntje**, **Karin** en **Aysegul** heel erg bedankt voor jullie inzet, steun en goede werken. Het is fijn om een maatje te hebben om mee te sparren. Eenieder van jullie heeft een grote bijdrage geleverd aan dit proefschrift maar eveneens aan de gezelligheid op de vakgroep.

Graag wil ik mijn twee 'afdeling-papa's **Frits Molenaar** en **Jan Visser** bedanken. Eerst als student, en later als AIO heb ik altijd even binnen kunnen stappen voor een praatje of advies. Frits, bedankt voor je interesse, steun en mooie verhalen. Ik vind het mooi en bijzonder hoe dicht je bij jouw gevoel staat. Het is wel een beetje stil geworden nadat je met pensioen gegaan bent; geen hard gefluit en muziek meer vanuit dat vertrouwde kamertje en ook geen 'hoi Liesje!' of 'hoi Liselotje!' meer. Ik wens je alle geluk toe, samen met je vrouw, familie en geiten. Jan, gelukkig heb ik jouw 'dag schoonheid!' niet hoeven missen. Bedankt voor de tijd die je in mij gestoken hebt om te HPLC-en, gezellig te borrelen, mijn rommel op te ruimen en mijn declaraties te redden als ik weer eens veel te laat was met inleveren (sorry daarvoor). Bij elke oranjebitter die ik zie, ruik of proef zal ik aan jou denken. Ik wens ook jou alle goeds toe samen met Riet, kinderen en kleinkinderen.

Andreia, Angela, Anne-miek, Ansar, Benoit, Bert, Christa, Gillian, Hans, Inge de Graaf, Inge Westra, Kaisa, Mackenzie, Magdalena, Marina, Marieke Elferink, Marjolijn, Martin, Ming, Miriam, Na, Patricia, Paul, Peter, Rose, Sanna, Suresh, Sylvia Blomsma, Sylvia Hummel, Venkatesh, Viktoriia, and Xiaoyu, I would like to thank you for your contribution to a warm and nice atmosphere. It was a pleasure working with you! Andreia, Benoit, Ming, Patricia, Suresh and Viktoriia, good luck with your PhD! Gillian, bedankt voor het regelen van heel veel dingen, je lachwekkende verhalen en je gezelligheid. Inge Westra, leuk dat ik je beter heb leren kennen in San Francisco. Bewaar fijne herinneringen aan ons tripje! Heel veel succes met het afronden van jouw PhD. Inge de graaf, bedankt voor alles en veel klus-en woonplezier in je nieuwe huis. Marina, ik heb van je aanwezigheid geno-

ten. Bedankt! Sylvia Hummel, tot aan het einde van mijn PhD heb ik bij jou in huis gewoond. Sorry dat ik niet een super gezellige huisgenoot voor je ben geweest en alleen maar werkend aan mijn burootje zat. Bedankt voor je interesse, aanmoedigingen en zeker ook je aanwezigheid.

Graag wil ik **kinderdagverblijf Pebbels** (ik noem het gewoon nog even zo) bedanken voor hun goede zorg. Lieve allemaal, met een heel goed gevoel hebben we altijd ons kostbaarste bezit bij jullie achtergelaten. Bedankt voor alle goede zorgen, interesse en flexibiliteit. Ook wil ik **kinderdagverblijf Rambamboelie** in Aruba bedanken. Miriam, Saskia en Jennie, jullie hebben Mathijs en Bram enorm goed ontvangen en opgevangen. Elke ochtend staan ze te springen als ze bij jullie mogen spelen. Dankjewel daarvoor!

Lieve buurtjes van de Vogelkersstraat, bedankt voor alle hulp en gezelligheid! Ik heb fijne herinneringen aan 'ons' straatje.

Gedurende de afgelopen jaren heb ik een aantal fijne en lieve mensen om mij heen verzameld, waar ik enorm trots op ben. Heel graag wil ik deze vrienden bedanken voor interesse, begrip, hulp, gezelligheid en voor alles wat ik niet eens op kan noemen. Lieve **Minke en Rinze**, bedankt voor de fijne momenten met z'n viertjes of samen met de kids. Ik vind het fijn dat we in Nederland zijn als jullie derde kindje wordt geboren, kunnen we de kleine gelijk in onze armen sluiten. Minke volgend jaar zijn we alweer 15 jaar vriendinnen; zullen we dat eens uitgebreid vieren? Lieve **Willemijn en William**, het is altijd op en top genieten als we met jullie zijn. Bedankt voor de fijne weekendjes weg, jullie heerlijke kookkunsten en de ongedwongen sfeer. Lieve **José**, ik zie ons nog samen pannekoeken bakken, mijn huis verven, struiken vervoeren op de bakfiets en zo kan ik nog we even doorgaan. Bedankt voor je trouwe vriendschap. Ik ben enorm trots op wat je allemaal doet en kan! Lieve **Janneke**, jij weet als geen ander wat het is om een promoverende mama te zijn, maar ook wat het is om een partner te hebben die alleen maar weekenden thuis is. Jullie hebben inmiddels een heerlijk plekje gevonden in het midden van het land en wij hebben binnenkort ook ons eigen stekkie op Aruba. Bedankt voor de gezellige lunches, afspraakjes en etentjes. Veel geluk samen met Cornelis, Coen en straks de kleine. Lieve **Bart**, ik zie je veel te weinig maar als ik je zie dan word ik meteen blij. Bedankt voor je vriendschap, gezelligheid en dat je altijd voor mij kiest als ik Djoek pest. Doe je wel voorzichtig op die motor van je? Veel plezier op je wereldreis! Ik wens je alle geluk toe, dat verdien je echt! Mijn lieve **FREEKjes; Ellen, Esther, Lucia, Nicole en Rianne**. Wat ben ik blij dat we elkaar zijn tegengekomen bij farmacie. Wat allemaal begon met kansloze kroegsessies is uitgelopen op een fijne, ongedwongen vriendschap. Ik vind het bijzonder dat we nu allemaal mama zijn en merk dat we rustiger en evenwichtiger zijn dan ooit tevoren. Hopelijk blijven we tijd voor elkaar vinden en houden we ons jaarlijkse event erin. Ik ben trots op al alles wat jullie voor elkaar hebben gebokst maar nog veel meer op jullie fijne persoonlijkheden!

Al heel wat jaartjes loop ik rond bij de Vogeltjes. Lieve **Frouk en Piet**, bedankt voor jullie oneindige interesse, zorg en liefde. Het is vast niet makkelijk om ouders te zijn van een stel vogeltjes dat overal maar heen fladdert. Gelukkig voelt het alsof jullie altijd dichtbij ons zijn. Lieve **Tamara en Roger**, heel erg bedankt voor jullie steun, zorg en gezelligheid! Ik ben super blij met en trots op jullie!

Mijn familie wil ik bedanken voor hun onvoorwaardelijke liefde, steun en begrip. Lieve **pap en mam**, jullie hebben voor mij een goede, liefdevolle basis gevormd waarop ik alles kan ondernemen en uitproberen. Ik hoop dat wij Mathijs en Bram datzelfde gevoel van vertrouwen kunnen geven. Heel erg bedankt voor het steunen van al mijn keuzes en voor de goede zorgen over ons alle vier. Lieve **Erik en Manon**, **Jeroen en Tessa**, **en Irene**, bedankt dat jullie er altijd voor mij en mijn gezinnetje zijn. Met veel liefde ontfermen jullie je over onze jongetjes, wat mij een veilig gevoel geeft. Ik ben trots op jullie alle 5! Irene, ik ken niemand met zoveel doorzettingsvermogen als jij. Door jouw gevecht tegen die stomme MS besef ik mij dag in, dag uit dat niets vanzelfsprekend is. Al wonen we nu misschien ver weg, we zijn altijd dichtbij je. Onthoud dat goed. Lieve **Vana, Mika, D-Jayson, Esmee, Lizzy, Solènn en Yulia**; in vind jullie echt super lief en ben reuze trots om jullie tante te zijn!

Tot slot wil ik mijn lieve jongens **Djoek, Mathijs** en **Bram** bedanken. Djoek, bedankt voor alle offers die je hebt gemaakt om mij te ondersteunen. Ik ben enorm trots op wie je bent, hoe je met onze jongens omgaat en wat je allemaal hebt bereikt. Ik heb zin in onze toekomst op Aruba. Mathijs en Bram, ik word zo vrolijk en glim van trots als ik jullie lieve snoetjes zie. Bedankt voor alle kusjes, knuffels, stoere verhalen en gezelligheid. Lieve kereltjes, ik wacht op de dag dat jullie dit kunnen lezen en hopelijk begrijpen dat ik mijn uiterste best doe om mijn ambities te combineren met het moederschap. Vergeet alsjeblieft nooit dat jullie mijn kostbaarste bezit zijn en ik er alles aan doe om jullie te laten stralen!

Cheers!

Marlies